A suite of selective pressures supports the maintenance of alleles of a *Drosophila* immune peptide

Running Title: Antagonistic roles of *Diptericin* alleles

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Abstract

The innate immune system provides hosts with a crucial first line of defense against pathogens. While immune genes are often among the fastest evolving genes in the genome, in *Drosophila*, antimicrobial peptides (AMPs) are notable exceptions. Instead, AMPs may be under balancing selection, such that over evolutionary timescales multiple alleles are maintained in populations. In this study, we focus on the *Drosophila* antimicrobial peptide Diptericin A, which has a segregating amino acid polymorphism associated with differential survival after infection with the Gram-negative bacteria *Providencia rettgeri*. Diptericin A also helps control opportunistic gut infections by common Drosophila gut microbes, especially those of *Lactobacillus plantarum*. In addition to genotypic effects on gut immunity, we also see strong sex-specific effects that are most prominent in flies without functional *diptericin A*. To further characterize differences in microbiomes between different *diptericin* genotypes, we used 16S metagenomics to look at the microbiome composition. We used both lab reared and wild caught flies for our sequencing and looked at overall composition as well as the differential abundance of individual bacterial families. Overall, we find flies that are homozygous for one allele of *diptericin A* are better equipped to survive a systemic infection from *P. rettgeri*, but in general have a shorter lifespans after being fed common gut commensals. Our results suggest a possible mechanism for the maintenance of genetic variation of *diptericin A* through the complex interactions of sex, systemic immunity, and the maintenance of the gut microbiome.

Introduction

An effective immune response is essential for organisms to protect themselves from pathogens. However, an excessive immune response causes damage to self either directly (autoimmunity, cytokine storms) or through dysbiosis *via* disruption of the composition of beneficial microflora [1–4]. The dynamic suite of microbes that hosts face necessitates initiating a robust systemic immune response, avoiding self-harm, and maintaining a beneficial microbiome. The challenge of maintaining a balanced immune response is exacerbated because the threat of microorganisms is contextual; thus, the immune system must distinguish harmful versus beneficial microbes in each context [5,6]. For example, an ingested microbe may be harmless or even beneficial as a food source in the digestive tract, but, when in circulation, that same microbe may prove harmful to the organism [7]. One consequence of balancing a robust innate immune system is that it may lead to the maintenance of genetic polymorphism in genes that encode for proteins intimately involved in the immune response [8,9]. Such patterns of maintained polymorphisms contrast the standard coevolutionary arms race model where hosts and pathogen continually adapt to each other leading to rapid evolutionary change in immune genes [10–12]. Though the coevolutionary arms race model appears apt in many cases, the case for the adaptive maintenance of alleles, balancing selection in the broad sense, on genes involved in the immune defense stems from a more nuanced view of the delicate interplay of systemic immunity, life history traits and the beneficial microbiome [13,14].

Balancing selection is the general term for the adaptive maintenance of multiple alleles in a population. In contrast to the evolutionary arms race model, natural variation in many immune genes is maintained by balancing selection [15–18]. The inference of balancing selection from

60 molecular population genetic data is difficult, especially when genes are small and in areas of
61 high recombination. Nonetheless, several examples of balancing selection exist, many of which
62 involve small effector proteins of the innate immune system [18–21].
63
64 Balancing selection on immune genes is likely to involve allelic benefits that are only
65 conditionally beneficial. One way that an allele could be conditionally beneficial is if there is
66 specificity between pathogen and allele [22] such that *allele A* better protects against *pathogen 1*
67 and *allele B* better protects against *pathogen 2*: *pathogen specificity hypothesis*. Another way that
68 an allele can be conditionally beneficial in the context of immune defense is if resistance alleles
69 are costly in the absence of infection. In this case, *allele A* better protects against both *pathogen*
70 *1* and *pathogen 2*, but in the absence of infection *allele A* is costly for its host [23]. This cost
71 could be energetic or through autoimmune-like damage: *autoimmune hypothesis*. In reality, there
72 is likely a continuum between these alternative hypotheses.
73
74 Invertebrates lack an adaptive immune system, so the delicate balance between systemic and gut
75 immunity is achieved only through adjustments to the innate immune response [24]. It is
76 therefore important to understand how invertebrates optimize their systemic immune response
77 with as little detriment to their beneficial gut microbiota as possible.
78
79 Though first characterized for its role in systemic immunity, the (IMD) pathway is also the main
80 NF-κB immune pathway in the gut and contributes to the maintenance of the microbiome and
81 protection from gut infections [25–27]. To date, there is little research into how individual
82 AMPs help maintain a healthy microbiome composition in the fly, let alone how allelic variation

may affect microbiome composition [28,29]. The standard gut microbiome of lab reared *D. melanogaster* consists of two prominent bacterial genera: *Lactobacillus* and *Acetobacter* [27]. Both genera are easily cultured in the lab, and the *Drosophila* gut can easily be manipulated, making it a good model to study the effects of immune gene variation on microbiome composition and how microbiome composition can, in turn, influence host fitness.

Antimicrobial peptides (AMPs) are a critical part of the innate immune system that act as broad-spectrum antimicrobials, combating bacteria, fungi, and viruses. In the coevolutionary arms race model, AMPs are on the front lines: directly interacting with microbes. AMPs have also been demonstrated to have critical roles in other physiological functions, for example, dysregulation of AMPs has been connected to diseases such as atopic dermatitis and Alzheimer's disease [2,30,31]. AMPs also contribute to the aging process where the immune system becomes more active as organisms ages to compensate for a decline in its effectiveness. However, this leads to cytotoxicity that in turn shortens lifespan [3].

In *Drosophila,* AMPs play a crucial in both manage systemic infections and in the maintenance of the gut microbiota [32]. AMPs, however, appear to evolve more slowly than most immune gene families [33–36]. One explanation for this perceived lack of adaptive evolution is that *Drosophila* AMPs genetic variation is maintained adaptively through balancing selection [15,37–39], where different selective pressures favor different alleles. Naturally occurring allelic variation in AMP loci are sometimes associated with variability in pathogen resistance [19,40–42]. *Diptericin A* (hereon referred to as *diptericin* or *Dpt)* is one of the canonical effector genes

105 of the *Drosophila* Immune deficiency (IMD) pathway, and is generally associated with defense
106 against Gram-negative bacterial infection [43,44].
107
108 In both *D. melanogaster* and *D. simulans*, an amino acid polymorphism at the 69th
109 residue of the mature 83-residue Diptericin peptide segregates in most populations surveyed
110 [19]. In both species, this polymorphism is due to a point mutation that changes the ancestral
111 serine (S) allele to arginine (R), but the two species use different codons for arginine. Unckless *et*
112 *al.* [19] found a significant difference in survival based on *diptericin* genotype after systemic
113 infection with the Gram-negative bacteria, *Providencia rettgeri,* a natural pathogen of *D.*
114 *melanogaster.* The study used inbred fly lines and found homozygous serine flies have a better
115 survival rate 5 days post infection (~60%) than homozygous arginine flies (~20%) or flies with a
116 premature stop codon in *diptericin* (0%). Later work by Hanson *et al.* took a more general
117 approach to AMP specificity and found that *Diptericin* plays a disproportionate role in response
118 to infection with *P. rettgeri [45].* What remains unclear, however is why this presumably
119 deleterious arginine allele persist in populations of two different species. We hypothesize that it
120 either protects against a different suite of pathogens or it is beneficial in the absence of infection
121 through some life history tradeoff.
122
123 This study aimed to test two hypotheses about the maintenance of genetic variation in *Diptericin.*
124 First, different *Diptericin* alleles might protect against different pathogens – we refer to this as
125 the *pathogen specificity hypothesis*. It would be supported if the arginine allele were to be
126 associated with higher survival than the serine allele in some infections, since the serine allele is
127 already clearly more protective against *P. rettgeri* [19,42]. In contrast, flies with the serine allele

128 might be generally better at surviving infection, but this may have a cost to the organism. We
129 refer to this as the *autoimmune hypothesis,* where a protective immune allele is associated with a
130 tradeoff that is costly in the absence of infection. We test whether there are tradeoffs between
131 systemic immunity and other life history traits – particularly traits related to the gut microbiome
132 utilizing genetically controlled lines for *Diptericin* alleles generated by CRISPR/Cas9-editing.
133 Flies were systemically infected with a panel of six different bacteria and homozygous serine
134 flies had a better 5-day survival with each bacterium. We then used axenic and gnotobiotic flies
135 to look at the lifespan of flies since AMP overexpression and microbial proliferation are
136 commonly observed in aging flies [46–49]. *Lactobacillus plantarum* is harmful to female flies
137 with non-functional diptericin, while homozygous arginine flies poly-associated with *L.*
138 *plantarum* and *A. tropicalis* had a longer lifespan than homozygous serine flies. In this way we
139 found evidence for a tradeoff between the ability to fight systemic infection and the ability to
140 control opportunistic gut infections.
141

142 **Results**

143 **A single amino acid change drastically influences survival after infection.**
144 Unckless et al. [19] found that in inbred lines from the *Drosophila Genetic Reference Panel*
145 (DGRP), flies homozygous for serine as position 69 of the mature Diptericin peptide survive
146 systemic infection with *P. rettgeri* much better than those homozygous for the arginine peptide at
147 the same position. This experiment showed only an association between immune defense and the
148 serine/arginine polymorphism since these inbred lines were on several different genetic
149 backgrounds. To control genetic background, we used CRISPR/Cas9 genome editing to create
150 both an arginine allele (single nucleotide change, dpt^{S69R}) and multiple null alleles (1 or 3 base

151 pair deletions, *Δdpt* flies), as well as control dpt^{S69} (serine at position 69 of the mature peptide) in

152 *diptericin* (Fig S1A, Table S1). The phenotype for our CRISPR/Cas9 edited flies showed striking

153 similarity to the inbred lines. In systemic infection challenges with *P. rettgeri,* dpt^{S69} flies are

154 better protected from infection than dpt^{S69R} flies (p= 5.42e-08) and *Δdpt* flies (p=6.46e-09, Fig

155 1A). Remarkably, for inbred lines

156 and CRISPR/Cas9 edited lines,

157 survival for five days post infection

158 with *P. rettgeri* for the serine allele

159 lines is 50-60%, with the arginine

160 allele is 10-20%, and less than 5%

161 for null alleles.

162

163 We next challenged the

164 CRISPR/Cas9 edited flies with

165 systemic infection using multiple

166 other Gram-positive and Gram-

167 negative bacteria to determine

168 whether the arginine allele (dpt^{S69R})

169 protects against some infections

170 better than the serine allele. Such a

171 finding would support the

172 hypothesis that allelic variation is

173 maintained by different alleles providing specific protection against different microbes.

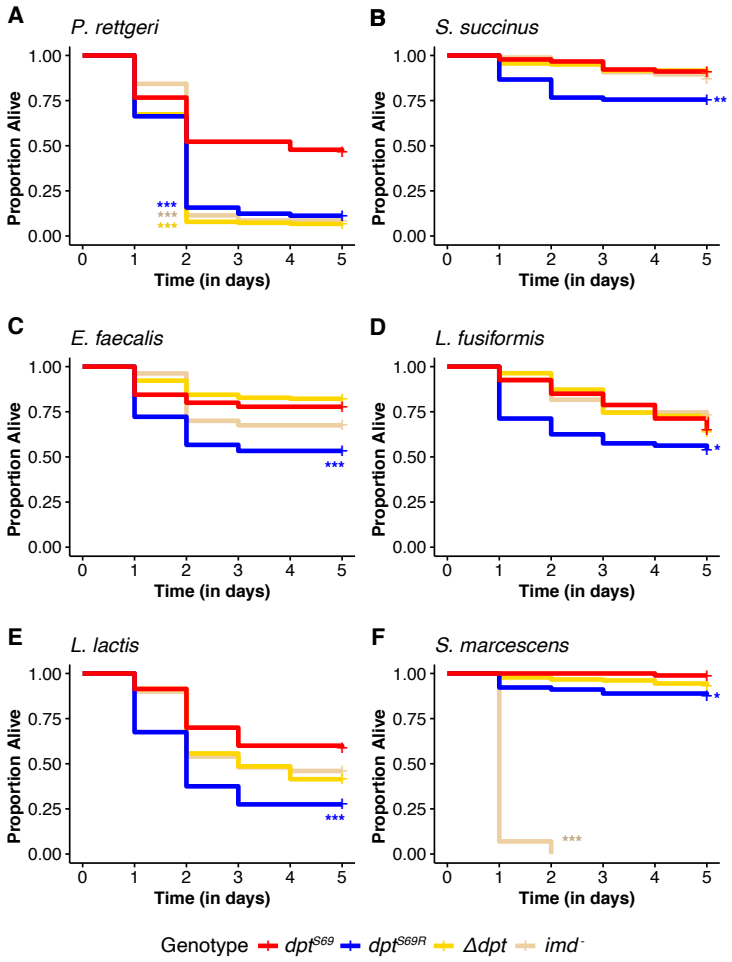

Figure 1: Five-day survival post systemic infection. Survival after systemic immunity challenge with the following bacteria A) *P. rettgeri,* B) *Staphylococcus succinus*, C) *Enterococcus faecalis*, D) *Lysinibacillus fusiformis* Strain Juneja, E) *Lactococcus lactis* F) *Serratia marcescens*. Each graph represents the combined results of 3 different infection dates of at least 20 males of each genotype for each date (at least 60 total). Significance is relative to dpt^{S69} (red line) using Cox proportional-hazards regression model. *P<0.05, **P<0.01, ***P<0.001.

174 However, the differential response to systemic infection with *P. rettgeri* at an OD_{600}=0.1, as
175 described above, remains the largest difference in immune response between *Dpt* genotypes.
176 Dpt^{S69} flies survived better than dpt^{S69R} flies for all systemic infections tested (Fig 1, Table S2).
177 In the Gram-positive bacterial infections (*Enterococcus faecalis*, *S. succinus, L. fusiformis,* and
178 *L. lactis)*, dpt^{S69R} flies had lower survival than that of *Δdpt* or imd^{-} (null allele for the Imd gene)
179 flies (Fig 1B-E). The only systemic infection where *dpt* genotype did not seem to matter was *S.*
180 *marcescens,* where all flies survive infection well except for imd^{-}, which die very quickly (Fig
181 1F).
182
183 We also tested if males and females showed differences in systemic immunity based on genotype
184 for *P. rettgeri, E. faecalis* and *L. plantarum* infections (Fig S2, Table S3). Generally, the *Dpt*
185 genotypes have qualitatively similar survival patterns between males and females when infected
186 with *P. rettgeri.* Females showed lower survival compared to males after systemic infection with
187 *E. faecalis* (p=0.0003). Higher male survival post infection was observed previously for both *E.*
188 *faecalis* and *P. rettgeri* with differences ascribed to the Toll pathway [50].
189
190 Overall, flies with the dpt^{S69} allele are better equipped to survive a systemic bacterial infection
191 than dpt^{S69R} flies, though this is most pronounced for *P. rettgeri*. Of the 6 bacteria tested, there
192 was no case where dpt^{S69R} flies survived the infection better than dpt^{S69} flies. These results do not
193 support the hypothesis that alleles are maintained to better combat different pathogens.
194

Diptericin genotype affects lifespan of mono- and poly- associated gnotobiotically reared flies.

Although our survey of systemic infections was not exhaustive, we did not find any instances where *dpt*^{S69R} flies were better able to fight infection, so we turned our attention to the role of *Dpt* in gut microbiome maintenance and immunity. The gut microbiome influences several life history traits in *Drosophila* and other organisms [51–54]. To dissect how *diptericin* genotype influences microbiome maintenance, we manipulated the microbiota in CRISPR/Cas9 flies, and measured longevity and bacterial load. We began with the longevity of axenically reared flies, since it represents a baseline survival without the presence of microbes. Flies with functional copies of *diptericin* (*dpt*^{S69} or *dpt*^{S69R}) did not differ in lifespan for either sex ($p_{genotype}$=0.3762), and *Δdpt* lines show a similar lifespan. However,

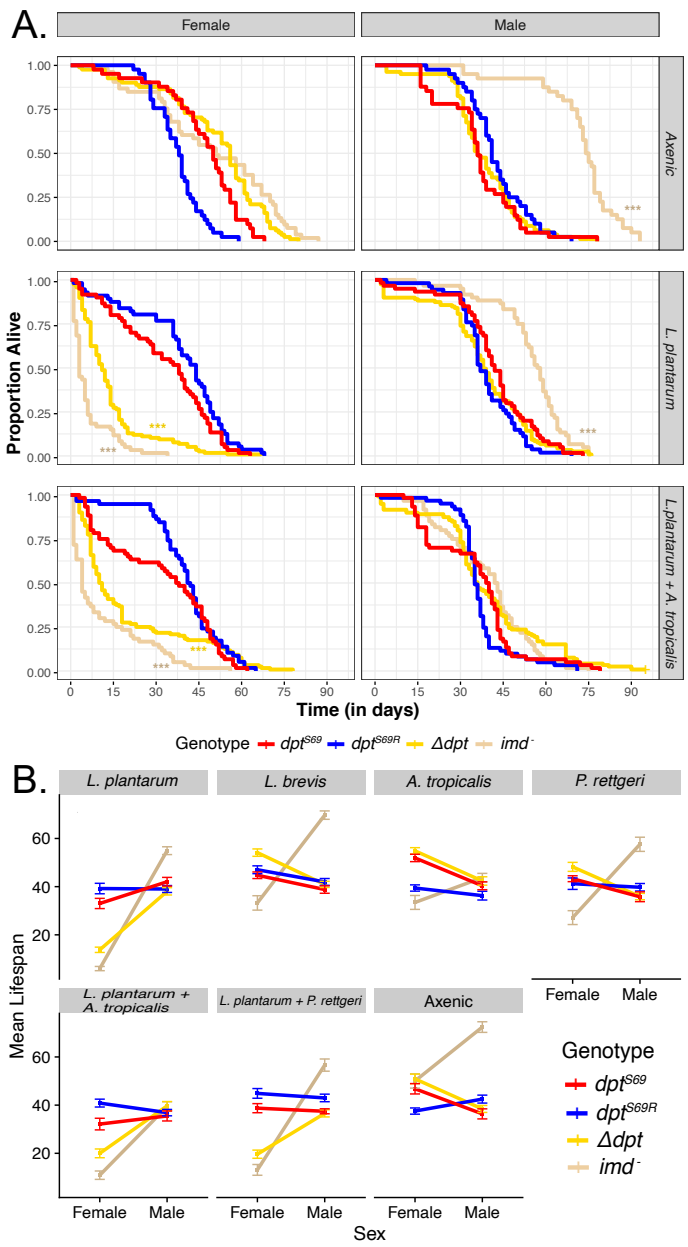

Figure 2: Axenic and gnotobiotic lifespan.
A) Survival plots for axenic flies and gnotobiotic flies mono-associated with L. plantarum or poly-associated with L. plantarum and A. tropicalis. Each curve represents 60 flies from 3 replicates of 20 flies. Significance is shown in comparison to SS (red line) using the model Lifespan~(Genotype*Sex)/Vial+Block. B) Mean lifespan was plotted against each sex and separated by bacterial association. Axenic flies have no bacterial association. Each point for each sex in a combination of 3 separate trials of 20 flies for a total of 60 flies. These interaction plots only have mean lifespan and thus are useful for seeing all the data at a glance. Error bars are mean plus or minus the standard error of the mean. *P<0.05, **P<0.01, ***P<0.001.

218 there was a difference in overall lifespan between the sexes among the CRISPR genome edited
219 lines (p_{sex}=1.04e-6). In *dpt*^{S69} or *Δdpt* female flies have a longer lifespan than male flies, as
220 observed generally for *D. melanogaster* previously [55]. In contrast, *imd*$^{-}$ male flies have a much
221 longer lifespan than any of the other lines tested (Fig 2A – axenic row, Table S3).
222
223 We next tested the influence of Diptericin genotype on mono-associations with standard
224 constituents of the *Drosophila* gut microbiome. We found multiple sex effects when axenically
225 reared flies were fed *L. plantarum, L. brevis,* or *A. tropicalis* (Figs 2A and 2B, S3). Flies that
226 were fed *L. plantarum* show the most striking differences. Male flies continued to show similar
227 lifespans to each other, but female *Δdpt* and female *imd*$^{-}$ flies both succumbed quickly post-
228 feeding, indicating functional *Diptericin* is important for gut immunity against opportunistic *L.*
229 *plantarum* in females.
230
231 We also fed axenic flies *P. rettgeri,* which does not readily cause gut infection, to axenically
232 reared flies. Recall that after systemic infection *dpt*^{S69} flies are more resistant to *P. rettgeri* than
233 *dpt*^{S69R} flies (Fig 1A). After monoassociation, however, we see no significant difference between
234 the two genotypes with *P. rettgeri* in either sex (p=0.612, Fig 2B). There is also no significant
235 difference between the *Δdpt* and lines with functional Diptericin (Fig 2B, S3). Surprisingly,
236 however, the null allele for *Imd* again shows a significant effect on survival in a sex-specific
237 manner: null *Imd* females die much earlier and null *Imd* males survive much longer when fed *P.*
238 *rettgeri.*
239

240 Some of the sex-specific differences in survival after monoassociation with bacteria may be
241 driven by intrinsic differences in feeding rates between the sexes (and potentially genotypes as
242 well) [56]. To determine whether the differences in male and female survival and load were due
243 in part to differences in feeding rates, we performed a feeding rate assay with blue dye mixed
244 with media (Luria-Bertani broth) or *P. rettgeri* (OD_{600}=15.0). We noted that females did eat
245 more in a single hour of feeding, but that there was also an effect of *diptericin* genotype (with
246 null flies eating less, Figure S4). Thus, it is possible that differences in longevity after
247 monoassociation are due to different rates of exposure to those bacteria because of different
248 feeding rates.
249
250 We next looked at how poly-associations with bacteria affected lifespan. First, we fed flies a 1:1
251 mixture of *L. plantarum* and *A. tropicalis*, two common gut microbes found in lab-reared and
252 wild caught flies [57,58]. We found that female *Δdpt* and imd^{-} flies had a much shorter lifespan
253 than flies with functional Diptericin (Fig 2A and 2B). This is the same pattern observed in mono-
254 association with *L. plantarum*. However, we observe that *diptericin* genotype influences survival
255 when poly-associated with *L. plantarum* and *A. tropicalis*. Dpt^{S69R} female flies live longer than
256 dpt^{S69} female flies (Fig 2A and 2B; p=0.00782). This is the opposite of systemic infections,
257 where dpt^{S69} flies always survived better than dpt^{S69R} flies, and may indicate a tradeoff between
258 defense against systemic and gut immunity.
259
260 In poly-association with 1:1 *L. plantarum* and *P. rettgeri,* we observe many of the same patterns
261 as in the poly-association with *L. plantarum* and *A. tropicalis*. Again, female *Δdpt* and imd^{-} flies
262 have a shorter lifespan than female dpt^{S69} and dpt^{S69R} flies (Fig 2B), and *dpt* genotype is

263 associated with lifespan. Dpt^{S69R} females have a longer lifespan than dpt^{S69} flies (p=0.000198,
264 Fig 2B).
265
266 Given the genotypic and sex effects on survival after oral association with *L.* plantarum, we also
267 looked at sex differences after systemically infecting conventionally reared flies with *L.*
268 *plantarum*. We saw no difference in survival between the sexes, as observed for systemic
269 infections with *E. faecalis* or *P. rettgeri* and saw no difference between *Δdpt* lines and lines with
270 functional Diptericin, as observed for axenic flies mono-associated with *L. plantarum* (Fig S2).
271 This could indicate that Diptericin plays different roles for systemic and gut immunity in relation
272 to *L. plantarum* in each sex. Further, dpt^{S69} male flies survived better than dpt^{S69R} male flies
273 (p=0.00438), in line with observations from other systemic infections in males (Fig 1).
274
275 Overall, we found a role of both specific *diptericin* genotype (serine vs. arginine) and the
276 presence of functional copies of *diptericin* for survival after introducing common gut microbes in
277 controlled conditions. Most striking was the sexually dimorphic role of both *Dpt* and *Imd-,* with
278 females being much more sensitive to genotype than males.
279
280 **Gnotobiotic fly bacterial load**
281 Given the differences in survival among sexes and genotype for different gnotobiotic
282 associations with bacteria, we determined whether bacterial load after associations also were
283 different among sexes and genotypes. To assess the influence of genotype on the immune
284 response of aging flies, we studied how well common gut bacteria colonized the gut over 20
285 days, representing ~20-33% of *D. melanogaster's* normal lab lifespan [59,60]. We generated

gnotobiotic flies by feeding specific bacteria for two days and observed the bacterial load 2-, 10- and 20-days post feeding.

When specifically looking at mono-association with *L. plantarum* we observe that bacterial load differences between genotypes occur within the first 2 days post feeding but begin to disappear by day 20 ($p_{genotype(day2)}$=2.6e-5, $p_{genotype(day20)}$=0.709, Fig 3A, S5). Note that in the first two days, the flies were raised on microbe-contaminated media, but after 2 days were moved onto sterile food and then transferred to new sterile food every 3 days. This corroborates what we saw in the longevity data in females. Within the first 15 days a large proportion of *Δdpt* female flies died, and we observed a higher bacterial load in these flies, especially on day 2 post feeding (Fig 2). By day 20, differences in bacterial load in females disappeared. Note that there is inherent sampling bias, as only the flies able to survive until day 20 are sampled at that time point. In the case of *imd*$^{-}$

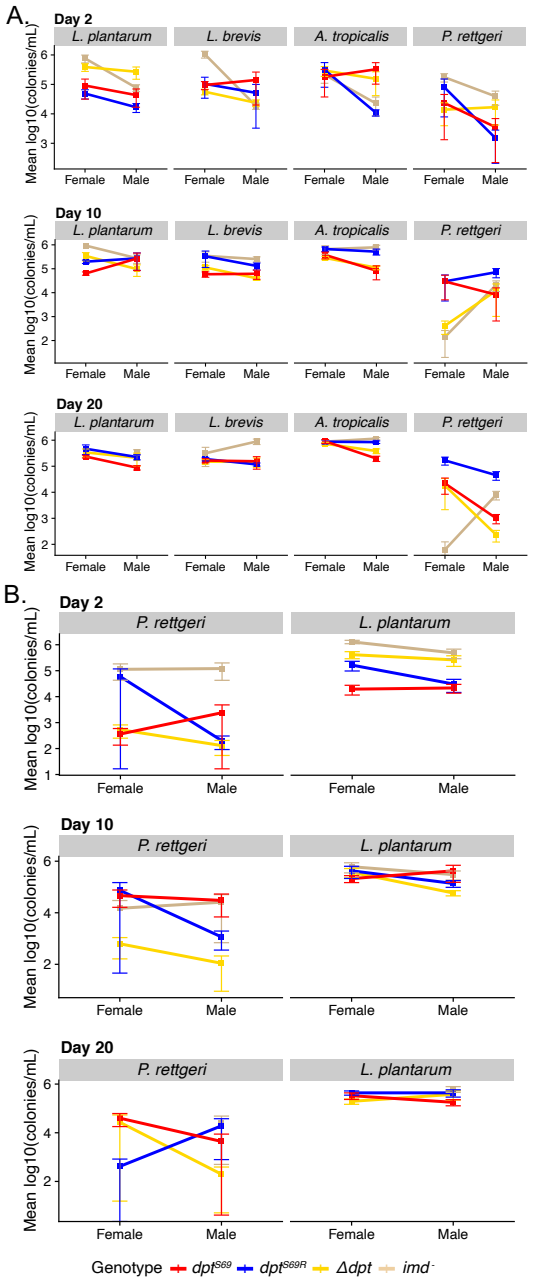

Figure 3: Bacterial load interaction plots for day 2, 10, and 20 post association with various microbes. Two-day old flies were placed on food seeded with a bacterial suspension of each bacteria at an OD600 of 15. Each point for each sex is from 3 separate trials of 2 samples of 3 flies each for a total of 6 samples per point. A) monoassociations where each plot represents a different condition, B) polyassociations where each row plots both the *P. rettgeri* and *L. plantarum* load from the same flies but were plated on LB and MRS respectively. Error bars are mean plus or minus the standard error of the mean.

308 flies, no females survived until day 20, hence there is no data for *imd*$^{-}$ flies on day 20 (Fig 3A,
309 S5).
310
311 *P. rettgeri* is the only canonical systemic pathogen we included in our gnotobiotic study, and the
312 patterns based on genotype were different between systemic infection and the gnotobiotic oral
313 infection data. When flies were fed *P. rettgeri,* we saw a range of bacterial loads, from zero
314 colonies to bacterial load levels on par with common gut bacteria. We also observed a genotype
315 effect between *dpt*^{S69} and *dpt*^{S69R} on day 10- and 20- post feeding in males ($p_{genotype(day10)}$=0.0044,
316 $p_{genotype(day20)}$=0.0004, model only included *dpt*^{S69} and *dpt*^{S69R}, Table S5). In both instances,
317 bacterial load in *dpt*^{S69R} flies is higher than in *dpt*^{S69} flies. This may indicate that *dpt*^{S69} flies are
318 better equipped to deal with both systemic and oral infection from *P. rettgeri*. Whether this is
319 due to a greater ability of *dpt*^{S69} flies to withstand the effects of infection (tolerance) by *P.*
320 *rettgeri* by remains a question. It is also important to note that *P. rettgeri* establishes poorly in
321 the gut of wildtype flies (personal observation), which may explain the noisy results for mono
322 associations after oral infection.
323
324 A range of bacterial loads were also observed when flies were poly-associated with *L. plantarum*
325 and *P. rettgeri* (Fig 3B, S6). There were no statistically significant differences between *dpt*^{S69}
326 and *dpt*^{S69R} bacterial for this poly association (Table S6). In fact, there were no significant
327 differences between any of the *P. rettgeri* bacterial loads, however *L. plantarum* does show
328 differences on day 2-post feeding (p=7.97e-6). These differences are mainly between flies with
329 non-functional *diptericin* (*imd*- and *Δdpt*) and flies with functional *diptericin*. Both *Δdpt* and
330 *imd*- flies had higher bacterial load than *dpt*^{S69} and *dpt*^{S69R} flies on day 2 post feeding which may

331 be an indication of the reason both these lines quickly succumb to feeding with *L. plantarum* at
332 least in the context of the poly-association with *P. rettgeri*.
333
334 We observed larger differences in bacterial load shortly after feeding with bacteria and those
335 differences became less by day 20-post feeding. We did not see any differences in *P. rettgeri*
336 load when mono-associated or when part of a poly-association, indicating the flies respond
337 differently to the same pathogen when introduced systemically or orally.
338
339 **Evidence for life history tradeoffs mediated by *diptericin* genotype**
340 Proteins involved in a robust immune response may have pleiotropic effects on other traits either
341 because of a direct interaction with that trait or because of inherent costs of immune defense
342 (self-damage, energy expenditure, etc.) [61–63]. We examined three life history traits
343 (desiccation stress survival, starvation stress survival, and uninfected longevity) to determine
344 whether *Dpt* genotype had such pleiotropic effects.
345
346 The ability to survive desiccation stress is an important life history trait for wild *Drosophila*
347 survival [64,65]. When subjecting our male CRISPR/Cas9 flies to desiccation, we observe
348 conventionally reared flies succumb faster to desiccation stress than axenically reared flies (Fig
349 4A, $p_{genotype} < 2e\text{-}16$). In conventionally reared males, dpt^{S69} flies have similar desiccation
350 resistance to dpt^{S69R} flies (p=0.917). However, dpt^{S69} flies survive desiccation stress better than
351 dpt^{S69R} flies when reared axenically ($p_{genotype}$=0.04126). We also compared *Drosophila* OreR and
352 W1118 (both homozygous for the serine allele of Diptericin) to the dpt^{S69} line (Fig S7, Table S7).
353 Unsurprisingly, despite the same *diptericin* genotype, all 3 lines show dramatically different

354 desiccation resistance. Therefore, *diptericin* genotype plays limited, if any role in variation in

355 desiccation resistance, as all 3 wildtype lines were derived from different genetic backgrounds.

356

357 We next looked at the effect

358 of genetic variation in

359 *diptericin* on male ability to

360 survive under starvation

361 stress. As with desiccation

362 resistance, we found

363 axenically reared flies

364 survive starvation stress

365 better than conventionally

366 reared flies (Fig 4B, S8,

367 $p_{treatment} < 2e\text{-}16$). However,

368 unlike the desiccation stress

369 conditions, both

370 conventionally reared and

371 axenically reared dpt^{S69R}

372 flies survive starvation

373 stress longer than dpt^{S69}

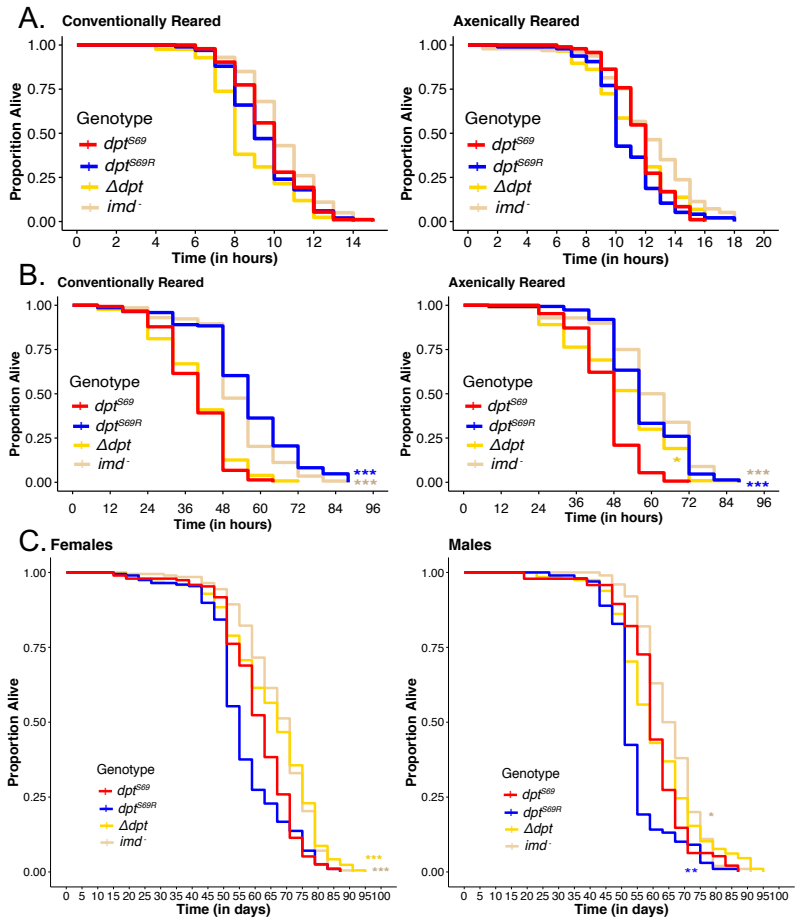

Figure 4: *Dpt* genotype is associated with variation in some life history traits

A) Conventionally and axenically reared male flies desiccation resistance (N>90 for each line apart from *Δdpt* line). There is no significance between dpt^{S69} (red line) and other genotypes. B) Conventionally and axenically reared male flies starvation resistance (N>90 for each line apart from *Δdpt* line). C) Female and male lifespan for conventionally reared flies. N>93 for each line and sex. Significance is shown in relation to dpt^{S69} (red line). Note that axenic longevity is shown in Figure 2. *P<0.05, **P<0.01, ***P<0.001.

374 flies (conventional: $p_{genotype} < 2e\text{-}16$, axenic: $p_{genotype} < 2e\text{-}16$). In conventionally reared flies

375 there is no difference in survival between dpt^{S69} flies and *Δdpt* flies (Fig 4B, Table S8). However,

376 in axenically reared flies, *Δdpt* flies have an intermediate survival phenotype between dpt^{S69} and

377 *dpt*^{S69R} flies. This may suggest an interaction between functional *diptericin* and the microbiome
378 that influences starvation.
379
380 Finally, we looked at overall longevity of female and male conventionally reared flies (in the
381 absence of any infection or other significant selection pressure). Female flies have a longer
382 lifespan than males (Fig 4C, S9, p_{sex}= 5.80e-13) regardless of *dpt* genotype. In males, *dpt*^{S69} flies
383 had a significantly longer lifespan than male *dpt*^{S69R} flies (mean of 60.3 days for *dpt*^{S69} and 54.1
384 for *dpt*^{S69R}, p= 0.0072), but not in female flies (mean of 61.9 days for *dpt*^{S69} and 59.0 for *dpt*^{S69R},
385 p= 0.6434). However, in axenically reared flies only female *dpt*^{S69} flies have a longer lifespan
386 than male *dpt*^{S69R} flies. Interestingly, the female flies with the longest lifespan have non-
387 functional Diptericin (Fig 4C, *Δdpt* line and *imd*$^{-}$ line, Table S9). Males lacking functional
388 Diptericin show the same effect, but to a lesser extent. These results provide evidence that, in the
389 presence of a standard gut microbiota, both *Dpt* and a functional Imd pathway may decrease
390 longevity. This is consistent with others who found downregulation of NF-κB pathways and
391 AMPs increased lifespan in *Drosophila* [63,66], but the fact that flies with *Dpt* null alleles alone
392 are sufficient to increase lifespan is noteworthy.
393
394 **Diptericin's influence on gut microbial diversity**
395 To determine whether *Diptericin* genotype influences the composition of the bacterial
396 community in the gut, we sequenced amplicons of 16s ribosomal rRNA in conventionally reared
397 lab flies under two different rearing conditions: flies reared in standard *Drosophila* vials, and the
398 progeny of the cross between *dpt*^{S69} and *dpt*^{S69R} flies reared in cages for more than 2 generations.
399 The vials may capture the long-term impact of genotype on microbiota but makes ruling out

stochastic changes in communities difficult. The cages ensure identical microbiota during development, but do not allow gradual effects of genotype on microbiota to accumulate. First, we found flies that were co-reared in cages had similar microbiomes, with no discernable differences by the alpha diversity metric, Shannon diversity (p=0.5239, Fig 5A). On the other hand, the microbiomes of flies reared in vials was distinctly different overall compared to the microbiomes of flies reared in the cages (p<0.001, Fig 5B). However, the differences between genotypes were still minimal in the vial-reared flies and vial may be a large factor in differences between lines. There were 2 dpt^{S69R} lines used and even though these are the same genotype and genetic background there was a significant difference between the 2 lines (Fig 5B). This difference is likely due to the within-vial drift in gut microbiome communities [67], which we attempted to control for using flies of different genotypes reared in the same cage (Fig 5A).

The lab is a controlled environment that allows for excellent control of variables but

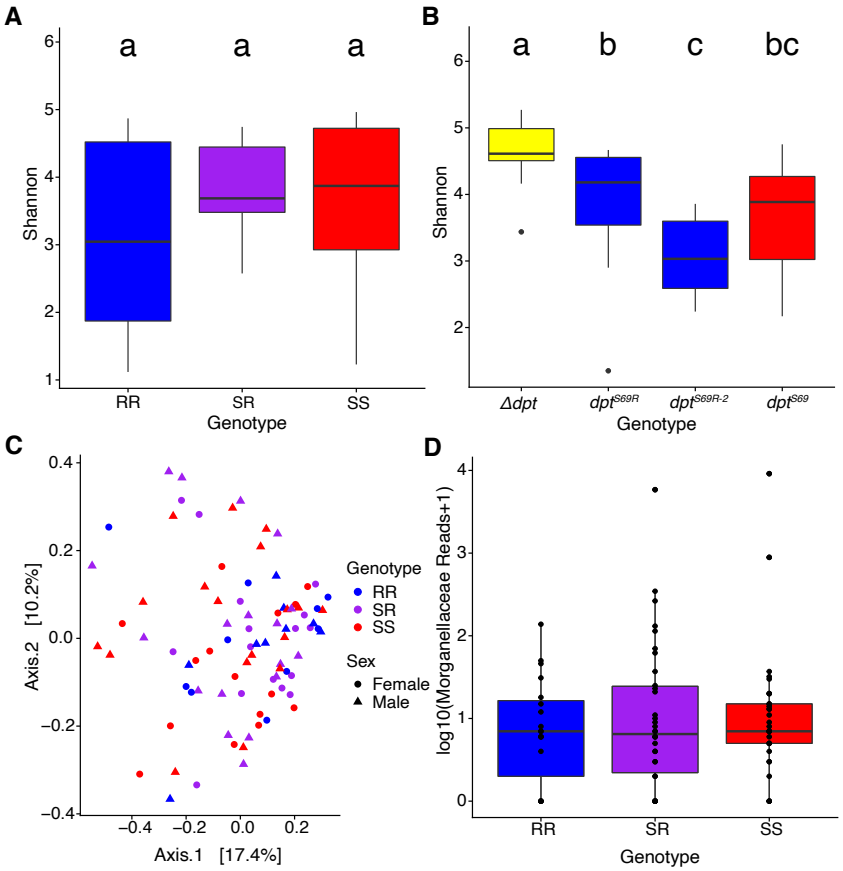

Figure 5 The influence of *Dpt* genotype on the gut microbiota
A) Shannon diversity of flies co-reared in cages. B) Shannon diversity of individual genotypes reared in vials. C) Bray-Curtis dissimilarity of wild caught flies. D) Differential abundance of Morganellaceae in wild caught flies. Different letters show significance based on post-hoc Tukey test, P<0.05.

423 does not replicate the conditions found in nature. Thus, we looked at the microbiomes of wild
424 caught flies collected from the decaying fruit of an apple orchard. We found flies with
425 homozygous for dpt^{S69R}, homozygous for dpt^{S69}, and heterozygous genotypes at our collection
426 location (Table S10). Out of the 955 *D. melanogaster* flies successfully genotyped, only 20 flies
427 were homozygous for dpt^{S69R} were identified. All 20 homozygous dpt^{S69R} flies were profiled
428 along with 36 each of dpt^{S69} homozygous flies and heterozygous flies for a total of 92 flies
429 profiled (amplicon sequences of both *Dpt* and 16s rRNA are in Table S2).
430
431 We found that *dpt* genotype does not affect the overall composition of the microbiome of wild
432 caught flies based on Shannon diversity (alpha diversity) and Bray-Curtis dissimilarity (beta
433 diversity) (Fig 5C). However, when looking at differential abundance of individual bacterial
434 families there are differences based on genotype. We tested specific association between
435 *diptericin* genotype and normalized counts from 9 microbial families. Most intriguing is the
436 difference in abundance of *Morganellaceae* family reads since, *P. rettgeri* belongs to this family.
437 Heterozygous and homozygous serine flies have a slightly higher abundance than homozygous
438 arginine flies (Fig 5D), but these differences were not significant.
439
440 Overall, our results for associations between *Dpt* genotype and gut microbiome in both lab and
441 wild flies are weak. Such associations may require much larger sample sizes if the effects are
442 small – particularly given the noisy phenotype in the wild. Alternatively, the influence of *Dpt*
443 genotype on gut microbiome diversity may be specific to developmental life stages or microbes
444 that are relatively rare in these populations.
445

446 **Discussion**

447 The adaptive maintenance of multiple alleles of a single gene has been posited for nearly a
448 century [68–72] and evidence for its pervasiveness continues to grow [73–78]. However, there
449 are relatively few cases where we understand the adaptive benefits of both allele (sickle-cell
450 anemia and malaria resistance being the best studied example [79]). In this study, we generated
451 CRISPR/Cas9 genome-edited flies of different *diptericin* genotypes to investigate mechanisms of
452 maintenance of allelic variation in AMPs. This allowed us to specifically study a single amino
453 acid change on an otherwise genetically-controlled background. Overall, we found evidence that
454 dpt^{S69} flies survive systemic infection better, while dpt^{S69R} flies survive some opportunistic gut
455 infections better. Thus, more robust defense against systemic infection appears to interfere with
456 maintaining a balanced gut microbiota – this tradeoff may result in natural populations
457 maintaining both alleles. Importantly, we note that while our results are consistent with the
458 adaptive maintenance of alleles in natural populations, these lab assays do not prove the adaptive
459 value of different alleles in the field.
460

461 Systemic infections with *P. rettgeri* in CRISPR/Cas9 genome-edited lines confirmed the amino
462 acid polymorphism in Diptericin as the basis for the difference in immune response of flies with
463 different *diptericin* genotypes previously hypothesized *via* an association study [19].
464 Surprisingly, dpt^{S69} flies had a higher survival five days post infection than dpt^{S69R} flies in *all*
465 systemic infections tested. This may mean either that the serine allele provides improved defense
466 against all the tested systemic infections, or that alterations to other aspects of fly physiology
467 (notably the gut microbiota) could predispose arginine flies to poorer survival. It is also possible
468 that some of *diptericin's* influence on survival is mediated by changes in the microbiome. We

469 did not perform systemic infections on axenically-reared flies, but if *Dpt* genotype influences the
470 microbiome, which in turn influences survival after infection with other pathogens, *Dpt* should
471 not influence survival after systemic infection if flies are reared axenically.
472
473 Our study also reveals an important role of Diptericin in preventing opportunistic gut infections
474 by common gut microbes. This is especially evident when looking at lifespan after associating
475 axenic flies with *L. plantarum* in both mono- and poly- association contexts. In both sexes,
476 dpt^{S69R} flies have a longer lifespan than dpt^{S69} flies, and this effect is enhanced in poly-
477 association with *L. plantarum* and *A. tropicalis.* Other studies have posited a role for individual
478 AMPs in the maintenance of gut microbes in *Drosophila* [29], but this appears to be the first
479 example linking a functional copy of a single AMP to survival differences.
480
481 One of our most striking and unexpected results was the distinct sex differences in longevity
482 after association with common gut bacteria. Furthermore, fly genotype plays a significant role in
483 these differences. After association with *L. plantarum*, *imd-* females had the shortest mean
484 survival, while *imd-* males had the longest mean survival. The effect for *Δdpt* was similar:
485 females had much shorter average survival than the other two *dpt* alleles, but the three alleles
486 showed equivalent longevity in males. While the *imd-* sexual dimorphism is relatively consistent
487 across experiments with different microbes, the *Δdpt* effect seems to be more limited to
488 associations involving *L. plantarum.* This result not only highlights the importance of using both
489 sexes in microbiome research, but also confirms that functional Diptericin is important for
490 *Drosophila melanogaster* gut health. There is a growing body of literature on the effects of
491 sexual dimorphisms in immune response, but it is lacking for sexual dimorphisms in gut

492 immunity and our findings only emphasize the need to fill this gap (*Drosophila* immunity sexual
493 dimorphisms reviewed in Belmonte et al. 2020; also see [80]). Our feeding assay provides some
494 evidence that the differences between male and female survival after exposure to microbes is due
495 the amount of food (and therefore the number of microbes) consumed, but these differences
496 should be examined in much more detail. More generally, the sex by genotype effect suggests a
497 likely explanation for the maintenance of genetic variation: different fitness effects of alleles in
498 the different sexes [81]. Note that while our study focuses on the serine/arginine polymorphism,
499 at least 6 null alleles of *dpt* segregate in natural populations of *D. melanogaster* and the
500 frequency of those nulls shows clinal variation in both North American and Africa [82], thus the
501 nulls are likely maintained selectively too.
502
503 Finally, we note that we have framed our argument in terms of balancing selection in the broad
504 sense because we are agnostic as to the population dynamics upon which these different selective
505 pressures might act. For example, it may be that some populations are disproportionately
506 infected by *P. rettgeri* or other microbes for which the serine allele is favored, while other
507 populations are relatively pathogen-free and the arginine allele is favored. This scenario is partly
508 supported by the evidence of clinal patterns in allele frequency in Africa and (to a lesser extent)
509 North America [83]. Similarly, these selective pressures could vary temporally. On the other
510 hand, these pressures could act all in one population where serine is favored in infected
511 individuals and the arginine allele is favored in uninfected individuals.
512
513 Herein we highlight 3 main roles of Diptericin in *Drosophila*. 1) Diptericin genotype influences
514 systemic immune defense; 2) in certain conditions Diptericin genotype influences gut immunity;

515 and 3) Diptericin has sex-specific effects in the gut. These results highlight the need for
516 individuals and populations to modulate the immune system to balance systemic and gut
517 immunity, and how the different needs of females and males complicate this balance. The
518 dramatic differences in survival of males and females in response to oral infection by common
519 gut bacteria underlines the importance of looking at both sexes when examining the maintenance
520 of genetic diversity, as balancing selection may be caused by sexual dimorphism. Overall, our
521 results suggest that a complex interaction between sex, environmental context (starvation,
522 pathogen exposure – both systemic and oral), and genotype may contribute to the long-term
523 maintenance of immune alleles.
524

525 **Materials and Methods**

526 **Drosophila Lines and Rearing**

527 Conventionally reared flies were maintained in a 23°C incubator with a 12h light:12h dark
528 schedule on a cornmeal-molasses-yeast diet (64.3g/L cornmeal, 79.7mL/L molasses, 35.9g/L
529 yeast, 8g/L agar, 15.4mL of food acid mix (50mL Phosphoric Acid + 418mL Propionic Acid +
530 532mL deionized water) and 1g/L Tegosept. We used CRISPR/Cas9 genome editing to modify
531 the *diptericin A* gene. Briefly, DNA coding for guide RNA (gRNA) was inserted into the pUS-
532 BbsI plasmid (Table S1). A single stranded donor DNA (120bp) containing the desired edit (to
533 change from serine to arginine) and a silent mutated PAM site was synthesized by IDT
534 (Coralville, IA, USA). The plasmid and ssODN were then injected into Bloomington stock
535 #55821, which expresses Cas9 driven by the *vasa* promoter, by Genetivision, Inc. (Houston, TX,
536 USA). Individual flies developed from the injected embryos were collected and crossed with a
537 modified version of Bloomington stock # 7198 (a line with *CyO*/*Kruppel* balanced on the 2nd

538 chromosome, and *serrate*/*Dichaete* balanced on the 3rd chromosome). Our version, 7198^{A4}, was
539 provided by Stuart Macdonald and has the DSPR [84] A4 line's X chromosome instead of the
540 *w*[*] from the original 7198. The F1s were collected and again individually crossed with 7198^{A4}
541 for F2 crosses yielding individuals with a homozygous 2nd chromosome representing one of the
542 chromosomes carried by the original injected embryo. The Dpt gene was sequenced from the F2
543 cross progeny to determine whether edits occurred. This yielded several classes of alleles
544 including homozygous serine *dpt* (wildtype, dpt^{S69}), homozygous arginine *dpt* (dpt^{S69R}), and *dpt*
545 null (*Δdpt,* refers to lines with either 1 or 3 base pair deletion) (Fig S1A). Balancers were
546 removed, and lines were moved into the same genetic background through a series of crosses as
547 shown in Fig S1B. An *imd*- line was used as an IMD pathway negative control but note that this
548 line was from a completely different genetic background from the rest of the lines.
549
550 **Axenic Fly Preparation**
551 Microbe-free (axenic) lines were generated by first washing embryos in a 10% bleach solution to
552 dissolve the chorion for 2 minutes. The embryos were then washed in 70% ethanol for 30
553 seconds and water for another 30 seconds, then transferred to autoclaved molasses food (see
554 above). Some embryos from each treatment were placed onto De Man, Rogosa and Sharpe
555 (MRS) agar plates and incubated at 30°C for 48 hours to check that they did not contain viable
556 microbes. Axenic lines were continuously checked for the presence of contaminating microbes
557 (every 3-4 generations) by homogenizing flies and plating the homogenate on MRS agar.
558
559 Axenically and gnotobiotically (see below) reared flies were maintained in an incubator that was
560 isolated from conventionally reared flies. The incubator was kept at 23°C with a 12h light:12h

561 dark schedule. Axenic and gnotobiotic flies were kept on the same molasses diet that had been
562 autoclaved before dispensing into autoclaved vials. Axenic and gnotobiotic flies were only
563 handled inside a sterile hood (Baker SG 400, The Baker Company Inc., Sanford, ME).

564

565 **Bacterial Strains**

566 The following bacteria were used for systemic infection assays: *Providencia rettgeri* [85],
567 *Providencia burhodegraneria* Strain B [85], *Enterococcus faecalis* [85]), *Serratia marcescens*
568 [40], *Lysinibacillus fusiformis* Strain Juneja [86], and *Staphylococcus succinus* (isolated from
569 wild *Drosophila,* Unckless lab). Bacteria were grown from glycerol stocks on LB plates at 37°C
570 overnight.

571

572 The following bacteria were used in gnotobiotic experiments: *L. plantarum, L. brevis, A.*
573 *tropicalis.* All these strains were isolated from plating conventionally reared flies on MRS agar
574 in the Unckless lab at the University of Kansas. Individual colonies from plated fly homogenate
575 were grown overnight in MRS for DNA isolation. Bacterial species were identified using Sanger
576 sequencing with the 16S rRNA region primers 27F (AGAGTTTGATCCTGGCTCAG) and
577 1492R (CGGTTACCTTGTTACGACTT). We also utilized the same *P. rettgeri* as described for
578 systemic infection.

579

580 **Survival Assays**

581 For systemic infections, individual colonies of bacteria were picked and grown in 2mL LB broth
582 shaking overnight at 37°C. Bacterial suspensions were then diluted or concentrated to OD_{600}=0.1
583 for *P. rettgeri,* OD_{600}=10 for *S. succinus,* OD_{600}=1.5 for *E. faecalis*, OD_{600}=3.5 for *L. fusiformis*,

584 OD_{600}=1.0 for *L. lactis*, and OD_{600}=4.0 for *S. marcescens*. *L. plantarum* was grown in 5mL
585 MRS at 30°C overnight and was concentrated to OD_{600}=10 for systemic infections. To induce
586 systemic infection, 5-9 day old, conventionally reared flies were pricked in the thorax with a
587 needle dipped in a bacterial suspension [87]. Infections were done in triplicate with at least 20
588 flies for each replicate per line for a total of 60 flies per genotype per condition. Flies were
589 incubated at 23°C with a 12h light:12h dark schedule and survival was tracked daily for 5 days
590 post infection.
591
592 **Gnotobiotic Longevity**
593 Axenically reared flies were collected within 24 hours of eclosion. Flies were then kept on sterile
594 food for 2 days before sorting for longevity experiments. To begin longevity experiments, flies
595 were separating into groups of 10 individuals of each sex and put onto sterile food seeded with
596 50uL of bacterial suspension at an OD600 of 15 +/- 1. For each replicate, we used 2 vials of 10
597 flies each per sex per line for a total of 20 flies for each sex per genotype for a total of 60 flies
598 across all replicates. Flies were allowed to feed in the inoculated vials for 3 days before being
599 transferred to uninoculated sterile food vials. Flies were flipped to new sterile media every 4-5
600 days for the remainder of the experiment. Surviving flies were counted every 1-3 days until all
601 flies were dead.
602
603 **Gnotobiotic Bacterial Load**
604 To determine whether microbes became established in the gut, we homogenized flies during and
605 after the exposure and plated the homogenate. We measured bacterial load by inoculating flies in
606 the same manner as gnotobiotic longevity. Flies were separated into groups of 5 females and 5

607 males per vial. For 2-day experiments flies were kept on the seeded food for the entire
608 experiment. For 10– and 20- day experiments, flies were allowed to feed on the seeded food for
609 3 days before being transferred to sterile food. Flies continued to be transferred to new sterile
610 media every 3-4 days until day 10 or 20 post feeding. After the experimental (2, 10 or 20 day)
611 time period, flies were surface sterilized by washing in 70% ethanol followed by molecular grade
612 water. Flies were separated by sex and three individuals were homogenized together in 300uL of
613 sterile 1x PBS and the homogenate was plated on the appropriate media using a Whitley WASP
614 Touch® spiral plater (Don Whitley Scientific, UK). When there were not 3 flies still alive then
615 all remaining flies of a sex were used and squished in 100uL of 1x PBS per fly collected instead
616 of 300uL to keep all samples the same concentration per fly. Counts were adjusted accordingly.
617

618 **Feeding Rate Assays**

619 To measure the amount of food and bacteria consumed by males and females of different
620 genotypes, we made food containing blue dye by adding 11.2g FCF blue dye (Erioglaucine
621 disodium salt) per liter of food. We used newly eclosed flies (1-2 days post eclosion / 14 days
622 post oviposition) and separated sexes (in sterile conditions) and keep flies at a density of 10
623 flies/vial in fresh food vials. We held these flies in incubators for one day, so they would recover
624 stress of anesthesia during sexing. To introduce bacteria (or control media) into the food, we
625 pipetted 50uL of suspension or LB at an OD600 of 15 (+/- 1) into each vial and allowed the
626 suspension to absorbed into the food for 30-40 mins by keeping the vials open inside a sterile
627 hood. We next added the experimental flies and allowed them to feed for one hour. After 1 hour,
628 we anesthetized the flies on ice, then rinsed in ethanol and sterile water. Flies were homogenized
629 in 300µl of 1X PBS with a glass bead (maximum speed for 4 minutes). The homogenate was

630 centrifuged at 14000 RPM for 4 minutes and 200µl of the supernatant was used to measure
631 absorbance at 630nm. Absorbance differences were analyzed using the natural log of absorbance
632 as the response variable with genotype, sex, genotype by sex interaction, and block as
633 independent variables. Due to the blocking structure of the experiment, each treatment (no media
634 control, LB control, *P. rettgeri*) was analyzed separately.
635
636 **Desiccation**
637 Desiccation survival assays were performed on dpt^{S69} and dpt^{S69R} adult males 4-7 days post-
638 eclosion in conventionally and axenically reared flies. Ten males were placed into an empty vial
639 and closed off with a cotton flug. Each genotype had 5 vials for a total of 50 flies per line per
640 rearing condition in each replicate. The flugs were topped with silica gel (Fisher, #S684) and
641 sealed with parafilm to prevent any moisture from entering the vials. Vials were kept at 24°C on
642 12 hour day/night cycles. Survival was measured by counting flies hourly until the entire
643 population died. This was repeated once more with 50 flies per line per rearing condition in each
644 trial for a total of 100 flies with the exception for the *Δdpt* line which in total only had 30 flies
645 per condition in total across trials.
646
647 **Starvation**
648 Starvation survival assays were performed on dpt^{S69} and dpt^{S69R} adult males 4-7 days after
649 eclosion for axenically and conventionally reared flies. Ten males were placed into a vial with
650 autoclaved starvation media (1% agar). The 1% agar was used to starve flies of nutrition but not
651 desiccate them. Vials were kept at 24°C on 12-hour day/night cycles. Survival was measured by
652 counting surviving flies at three 8-hour intervals (8 am, 4 pm, and 12 am) until all flies died. This

653 was repeated twice more for a total of 3 trials with at least 40 flies per line per rearing condition
654 in each trial for a total of at least 120 flies.
655
656 **16S Sequencing**
657 Flies were reared in the lab in two distinct ways for 16S rRNA sequencing of conventionally
658 reared flies. First, flies were taken from vials of individual genotypes. This allows for any
659 moderate effects of fly genotype to equilibrate over time. Second, flies were taken from cages
660 that were started with heterozygous *diptericin* flies. These cages were started with the F1
661 progeny from crosses of dpt^{S69} and dpt^{S69R} flies and allowed to continue for 3 discrete
662 generations before flies were collected for 16S rRNA sequencing. This ensures that the
663 genotypes are exposed to the same microbes and any differences in microbiome are due to
664 genetic differences manifest in that generation.
665
666 We isolated DNA from individual flies using Gentra PureGene Tissue DNA Isolation Kit
667 (Qiagen #158388, Qiagen, Germantown, MD) following manufacturer's instructions. DNA
668 pellets were rehydrated in 10 µl DNA hydration solution. Flies from cage populations were
669 genotyped by using the dpt_cw primer pair (Table S1) followed by a restriction enzyme digest
670 using enzymes that cut DNA sequences for arginine (BccI, NEB # R0704S) or serine (AluI, NEB
671 # R0137S).
672
673 DNA concentration was measured with Qubit fluorimeter (Invitrogen). Ten flies from each
674 inbred CRISPR genome edited line (5 females and 5 males from dpt^{S69}, dpt^{S69R}, $dpt^{S69R\text{-}2}$ (a
675 second homozygous arginine line from CRISPR/Cas9 genome editing), and *Δdpt* (1 base pair

676 deletion) and ten flies of each genotype from the cage population (5 females and 5 males of SS,
677 SR, RR genotypes) were each brought to a DNA concentration of 5 ng/µl. Libraries were
678 prepped in accordance with the 16S Metagenomic Sequencing Library Preparation protocol from
679 Illumina for the 16S V3/V4 region. Sequencing was performed on the Illumina MiSeq platform
680 using v3 300bp paired end reads. Library preparation and sequencing was performed at the
681 University of Kansas Genome Sequencing Core (Lawrence, KS, USA).
682
683 Wild flies were collected from decaying apples and pears in an apple orchard in Kansas City, KS
684 (3341 N 139th St, Kansas City, KS 66109). Flies were immediately transported back to the lab
685 and sorted by species on CO_2 and frozen at -20°C. DNA was extracted from individual flies
686 using Gentra PureGene Cell & Tissue DNA Isolation Kit (Qiagen #158388). The samples were
687 tested for species (*D. simulans* vs. *D. melanogaster*), *Wolbachia* status, and Dpt genotype using
688 primers listed in Table S1. Collections are summarized in Table S10. Libraries were prepped in
689 the same manner as the conventionally reared flies.
690
691 **16S Bioinformatic Analysis**
692 Demultiplexed reads were processed with QIIME2, v 2019.10 [88]. Primers were removed from
693 5' ends with Cutadapt using default parameters [89]. Reads were de-noised and trimmed for
694 quality with Divisive Amplicon Denoising Algorithm (DADA2) within the QIIME2
695 bioinformatics pipeline [90]. Forward and reverse reads were truncated at 280 bp and 245 bp
696 respectively. The remaining ASV table was exported from QIIME2 for further processing in R
697 [91]. Taxonomy was assigned to the ASV table using SILVA 16S rRNA gene reference
698 database, v.138 [92–94]. Reads assigned to Genus level *Wolbachia* (a *Drosophila* endosymbiont)

699 and Kingdom level Eukaryota were removed from further analysis. We also removed reads not
700 observed at least 3 times in at least 10 samples. Then, conventionally reared flies were rarefied to
701 17066 reads, while wild flies were rarefied to 10771 reads.
702
703 All statistical analysis of 16S data was performed in R (4.1.2) using the Phyloseq [95] package
704 and ggplot2 [96] package for visualization. The CRISPR and cage population data were analyzed
705 separately. For each population, alpha diversity was estimated using Shannon diversity in
706 Phyloseq using the estimate_richness() function. Bray-Curtis dissimilarity was calculated to look
707 at overall patterns of microbiome composition in Phyloseq using the ordinate() function.
708 Significance was determined at $\alpha=0.05$. Fixed effects models were fit with the package lme4 [97]
709 with the fixed effects genotype + sex + genotype*sex. When necessary, P-values were adjusted
710 for multiple comparisons using FDR correction method.
711
712 **Statistical Analysis**
713 R (version 4.1.2) was used to run statistical analyses. Survival data were plotted using the R
714 package survminer [98]. The analysis was performed using the Cox proportional-hazards
715 regression model in R [99]. For longevity, significance was determined using the model:
716 Lifespan~(Genotype*Sex)/Vial+Block. For gnotobiotic bacterial load, significance was
717 determined using the model: Colonies.mL~(Genotype)*Sex+Block.
718
719
720 **Competing Interests**
721 The authors state no competing interests.

722

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726 extractions of wild flies; and Elizabeth Everman for assistance with the feeding assay. The
727 University of Kansas Genome Sequencing Center Core (supported by NIH CMADP COBRE
728 P20-GM103638) performed 16s sequencing. SRM was supported by a University of Kansas Self
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730

731

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1002 **Supplemental tables**

1003 **Table S1. Oligonucleotide sequences used in this study.**
1004

Primer name	Primer sequence	Use	Reference
wsp-81F	TGGTCCAATAAGTGATGAAGAAAC	*Wolbachia* status	Braig et al. 1998
Wsp-691R	AAAAATTAAACGCTACTCCA	*Wolbachia* status	Braig et al. 1998
Actin79B-F	CGTCTACCAGTCCATCATGA	Species identification	
Actin79B-R	AGACGGAGTACTTGCGCTC	Species identification	
Dpt_cw_F	GCCTATAAAAGAGCATCGAA	*Diptericin*	Clark and Wang, 1997
Dpt_cw_R	GCTAGACTCGGATACCAATCGAG	*Diptericin*	Clark and Wang, 1997
Dpt_gRNA18_S	CTTCGATGGCAACTCAGAACCGACG	gRNA	This work
Dpt_gRNA18_A	AAACGCTCGGTTCTGAGTTCGGATC	gRNA	This work
ssODN	CGCCACGAGATTGGACTGAATGGAGG ATATGGACAGCACTTGGGAGGACCAT ACGGCAACTCAGAACCGAGATGGAA AGTGGGAAGCACCTACACCTACAGA TTTCCGAATTTCTAAGCT	ssODN	This work

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1006
1007
1008

1009 **Table S2. Cox proportional hazard pairwise significance p-values for systemic infection.** P-values less than
1010 0.05 are underlined.
1011

P. rettgeri			
	imd-	*Δdpt*	*dptS69R*
Δdpt	4.07E-02	-	-
dptS69R	4.54E-01	0.26329	-
dptS69	1.20E-04	1.10E-09	1.30E-06
S. succinus			
	imd-	*Δdpt*	*dptS69R*
Δdpt	0.4994	-	-
dptS69R	0.0672	0.0023	-
dptS69	0.4994	0.9828	0.012
E. faecalis			
	imd-	*Δdpt*	*dptS69R*
Δdpt	0.0264	-	-
dptS69R	0.0264	1.00E-06	-
dptS69	0.2969	0.3405	2.10E-03
L. fusiformis			
	imd-	*Δdpt*	*dptS69R*
Δdpt	0.435	-	-
dptS69R	0.048	0.22	-
dptS69	0.435	0.927	0.19
L. lactis			
	imd-	*Δdpt*	*dptS69R*
Δdpt	0.7515	-	-
dptS69R	0.0474	0.0474	-
dptS69	0.1966	0.0858	0.0015
S. marcescens			
	imd-	*Δdpt*	*dptS69R*
Δdpt	<2e-16	-	-
dptS69R	<2e-16	0.1151	-
dptS69	<2e-16	0.054	0.0042

1012
1013

1014 **Table S3. Cox proportion hazard ratio pairwise p-values for systemic infections with males and females.** P-
1015 values less than 0.05 are underlined.
1016

P. rettgeri							
	F-*imd-*	M-*imd-*	F-*Δdpt*	M-*Δdpt*	F- dpt^{S69R}	M- dpt^{S69R}	F- dpt^{S69}
M-*imd-*	0.2560	-	-	-	-	-	-
F-*Δdpt*	0.1592	0.8278	-	-	-	-	-
M-*Δdpt*	0.2356	0.9763	0.8278	-	-	-	-
F- dpt^{S69R}	0.9763	0.2981	0.1933	0.2560	-	-	-
M-*d* dpt^{S69R}	0.0144	0.1124	0.1510	0.1124	0.0152	-	-
F- dpt^{S69}	5.60E-05	0.0014	0.0014	0.0008	8.80E-05	0.3598	-
M- dpt^{S69}	3.70E-06	9.60E-05	9.60E-05	7.70E-05	6.50E-06	0.1639	0.5982
E. faecalis							
	F-*imd-*	M-*imd-*	F-*Δdpt*	M-*Δdpt*	F- dpt^{S69R}	M- dpt^{S69R}	F- dpt^{S69}
M-*imd-*	0.0304	-	-	-	-	-	-
F-*Δdpt*	0.0036	0.8954	-	-	-	-	-
M-*Δdpt*	0.0012	0.7414	0.7414	-	-	-	-
F- dpt^{S69R}	0.7414	0.1433	0.0326	0.0158	-	-	-
M- dpt^{S69R}	0.0318	0.9686	0.8893	0.7414	0.1566	-	-
F- dpt^{S69}	0.0326	0.9686	0.8779	0.7414	0.1701	0.9686	-
M- dpt^{S69}	0.0114	0.753	0.8664	0.9686	0.0511	0.7414	0.7414
L. plantarum							
	F-*imd-*	M-*imd-*	F-*Δdpt*	M-*Δdpt*	F- dpt^{S69R}	M- dpt^{S69R}	F- dpt^{S69}
M-*imd-*	<2e-16	-	-	-	-	-	-
F-*Δdpt*	3.80E-08	<2e-16	-	-	-	-	-
M-*Δdpt*	<2e-16	8.10E-09	<2e-16	-	-	-	-
F- dpt^{S69}	<2e-16	3.20E-09	7.60E-15	0.734	-	-	-
M- dpt^{S69R}	<2e-16	1.80E-12	8.30E-15	0.372	0.197	-	-
F- dpt^{S69}	<2e-16	6.00E-15	1.00E-10	0.08	0.056	0.548	-
M- dpt^{S69}	<2e-16	5.40E-07	<2e-16	0.364	0.662	0.056	0.013

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1020 **Table S4. P-values from Tukey HSD for axenic and gnotobiotic longevity.** P-values less than 0.05 underlined.

Axenic							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M- *dpt*^{S69R}
F-*Δdpt*	1	-	-	-	-	-	-
F-*dpt*^{S69R}	6.00E-04	<2e-16	-	-	-	-	-
F-*dpt*^{S69}	0.9447	0.8051	0.063	-	-	-	-
M-*imd*-	<2e-16	<2e-16	<2e-16	<2e-16	-	-	-
M-*Δdpt*	1.00E-04	<2e-16	1	0.0492	<2e-16	-	-
M-*dpt*^{S69R}	0.1713	0.0519	0.7467	0.8822	<2e-16	0.8133	-
M-*dpt*^{S69}	1.00E-04	<2e-16	1	0.0218	<2e-16	0.9947	0.5114
L. plantarum							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	0.0064	-	-	-	-	-	-
F-*dpt*^{S69R}	<2e-16	<2e-16	-	-	-	-	-
F- *dpt*^{S69}	<2e-16	<2e-16	0.185	-	-	-	-
M-*imd*-	<2e-16	<2e-16	<2e-16	<2e-16	-	-	-
M-*Δdpt*	<2e-16	<2e-16	0.9992	0.2479	<2e-16	-	-
M-*dpt*^{S69R}	<2e-16	<2e-16	1	0.2568	<2e-16	0.9999	-
M-*dpt*^{S69}	<2e-16	<2e-16	0.9531	0.0056	<2e-16	0.5593	0.9198
P. rettgeri							
	F-*imd*-	F-*Δdpt*	F- *dpt*^{S69R}	F- *dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	<2e-16	-	-	-	-	-	-
F-*dpt*^{S69R}	1.00E-04	0.1564	-	-	-	-	-
F-*dpt*^{S69}	<2e-16	0.5911	0.9977	-	-	-	-
M-*imd*-	<2e-16	1.12E-02	<2e-16	1.00E-04	-	-	-
M-*Δdpt*	2.34E-02	<2e-16	4.81E-01	9.95E-02	<2e-16	-	-
M-*dpt*^{S69R}	0.0013	3.63E-02	0.9997	0.9434	<2e-16	0.8429	-
M-*dpt*^{S69}	0.0938	1.00E-04	0.6415	0.2208	<2e-16	1	0.9079
L. brevis							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	<2e-16	-	-	-	-	-	-
F- *dpt*^{S69R}	<2e-16	0.0348	-	-	-	-	-
F- *dpt*^{S69}	4.00E-04	9.00E-04	0.9874	-	-	-	-
M-*imd*-	<2e-16	<2e-16	<2e-16	<2e-16	-	-	-
M-*Δdpt*	1.75E-02	<2e-16	1.18E-01	6.87E-01	<2e-16	-	-
M-*dpt*^{S69R}	2.06E-02	<2e-16	0.5139	9.63E-01	<2e-16	0.9998	-
M- *dpt*^{S69}	4.02E-01	<2e-16	0.0333	2.97E-01	<2e-16	0.9803	0.9241
A. tropicalis							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	<2e-16	-	-	-	-	-	-
F-*dpt*^{S69R}	0.2941	<2e-16	-	-	-	-	-
F-*dpt*^{S69}	<2e-16	0.9003	<2e-16	-	-	-	-
M-*imd*-	0.0016	<2e-16	0.6689	0.0255	-	-	-
M-*Δdpt*	0.0017	<2e-16	0.8677	4.00E-04	0.9987	-	-
M-*dpt*^{S69R}	0.9522	<2e-16	0.9472	<2e-16	0.0876	0.1404	-
M-*dptS69*	0.1403	<2e-16	1	2.00E-04	0.8812	0.9821	0.8103
L. plantarum + P. rettgeri							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M-*dpt*^{S69R}
F-*Δdpt*	0.1291	-	-	-	-	-	-
F- *dpt*^{S69R}	<2e-16	<2e-16	-	-	-	-	-
F- *dpt*^{S69}	<2e-16	<2e-16	0.3736	-	-	-	-
M-*imd*-	<2e-16	<2e-16	9.00E-04	<2e-16	-	-	-
M-*Δdpt*	<2e-16	<2e-16	0.0176	0.9872	<2e-16	-	-
M- *dpt*^{S69R}	<2e-16	<2e-16	0.998	0.807	1.00E-04	0.1564	-
M- *dpt*^{S69}	<2e-16	<2e-16	0.1446	0.9998	<2e-16	1	0.5051
L. plantarum + A. tropicalis							
	F-*imd*-	F-*Δdpt*	F-*dpt*^{S69R}	F-*dpt*^{S69}	M-*imd*-	M-*Δdpt*	M- *dpt*^{S69R}
F-*Δdpt*	0.008	-	-	-	-	-	-
F-*dpt*^{S69R}	<2e-16	<2e-16	-	-	-	-	-
F-*dpt*^{S69}	<2e-16	<2e-16	0.0632	-	-	-	-
M-*imd*-	<2e-16	<2e-16	0.9998	0.1957	-	-	-
M-*Δdpt*	<2e-16	<2e-16	0.9998	0.058	1	-	-
M-*dpt*^{S69R}	<2e-16	<2e-16	0.8864	0.7167	0.9893	0.9573	-
M-*dpt*^{S69}	<2e-16	<2e-16	0.6101	0.944	0.8792	0.7135	0.9997

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1022 **Table S5. P-values from Tukey HSD for bacteria load.** P-values less than 0.05 are underlined.

Day 2 L. plantarum							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.8875	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.9595	0.9999	NA	NA	NA	NA	NA
F-*dptS69*	0.4774	0.7296	0.6348	NA	NA	NA	NA
M-*imd-*	9.66E-01	1.00E+00	1	0.5932	NA	NA	NA
M-*Δdpt*	0.2644	0.1289	1.53E-01	0.9983	0.1308	NA	NA
M-*dptS69R*	0.8632	1	9.99E-01	0.924	0.9982	0.4745	NA
M-*dptS69*	0.8237	0.9999	9.96E-01	0.9628	0.9928	0.5926	1
Day 10 L. plantarum							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.8875	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.9595	0.9999	NA	NA	NA	NA	NA
F-*dptS69*	0.4774	0.7296	0.6348	NA	NA	NA	NA
M-*imd-*	9.66E-01	1.00E+00	1	0.5932	NA	NA	NA
M-*Δdpt*	0.2644	0.1289	1.53E-01	0.9983	0.1308	NA	NA
M-*dptS69R*	0.8632	1	9.99E-01	0.924	0.9982	0.4745	NA
M-*dptS69*	0.8237	0.9999	9.96E-01	0.9628	0.9928	0.5926	1
***Day 20 L. plantarum* (no imd females)**							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	NA	NA	NA	NA	NA	NA	NA
F-*dptS69R*	NA	0.9978	NA	NA	NA	NA	NA
F-*dptS69*	NA	1	0.9943	NA	NA	NA	NA
M-*imd-*	NA	0.9998	1	0.9991	NA	NA	NA
M-*Δdpt*	NA	9.35E-02	0.0314	0.2254	0.0553	NA	NA
M-*dptS69R*	NA	1.00E+00	0.9807	1	0.9952	0.3207	NA
M-*dptS69*	NA	3.40E-01	0.1438	0.5173	0.2122	1	0.6305
Day 2 P. rettgeri							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.0406	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.5572	0.9645	NA	NA	NA	NA	NA
F-*dptS69*	0.0234	0.9952	0.7777	NA	NA	NA	NA
M-*imd-*	0.5805	0.9565	1	0.7576	NA	NA	NA
M-*Δdpt*	2.10E-03	0.912	0.4482	1	0.4228	NA	NA
M-*dptS69R*	1.00E-03	0.5392	0.1887	0.9679	0.1757	0.9842	NA
M-*dptS69*	4.00E-04	0.3479	0.1066	0.8962	0.0984	0.9237	1
Day 10 P. rettgeri							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9999	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.1574	0.1425	NA	NA	NA	NA	NA
F-*dptS69*	0.9844	0.998	0.5859	NA	NA	NA	NA
M-*imd-*	0.0707	0.0541	0.9999	0.351	NA	NA	NA
M-*Δdpt*	0.9334	0.9767	0.57	1	0.3138	NA	NA
M-*dptS69R*	0.0086	0.0042	0.9253	0.0653	0.9903	0.0432	NA
M-*dptS69*	0.904	0.958	0.8201	0.9999	0.5938	1	0.1563
Day 20 P. rettgeri							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	1	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.0241	3.00E-04	NA	NA	NA	NA	NA
F-*dptS69*	0.7316	0.4202	0.1779	NA	NA	NA	NA
M-*imd-*	0.3846	0.0835	0.5982	0.9932	NA	NA	NA
M-*Δdpt*	1	1	3.00E-04	0.4122	0.081	NA	NA
M-*dptS69R*	0.0838	0.0032	0.996	0.5558	0.9479	0.003	NA
M-*dptS69*	0.9304	0.8325	0.0445	0.9984	0.8519	0.826	0.2147

Table S5 (continued)

Day 2 L. brevis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.0027	NA	NA	NA	NA	NA	NA
F-*dptS69R*	2.09E-02	1	NA	NA	NA	NA	NA
F-*dptS69*	0.0855	0.9932	0.9993	NA	NA	NA	NA
M-*imd-*	3.00E-04	0.8295	0.8593	0.5336	NA	NA	NA
M-*Δdpt*	<02e-16	6.19E-01	0.7332	0.3352	1	NA	NA
M-*dptS69R*	<02e-16	0.0511	0.1029	0.026	0.8176	0.7015	NA
M-*dptS69*	1.00E-04	0.5769	0.6552	0.3136	1	0.9999	0.9518
Day 10 L. brevis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9892	NA	NA	NA	NA	NA	NA
F-*dptS69R*	1	0.9836	NA	NA	NA	NA	NA
F-*dptS69*	0.6678	0.9554	0.6632	NA	NA	NA	NA
M-*imd-*	0.9941	0.6931	0.9989	0.2425	NA	NA	NA
M-*Δdpt*	8.49E-02	0.2951	0.1117	0.9918	0.0087	NA	NA
M-*dptS69R*	0.9987	1	0.9971	0.9634	0.8893	0.4734	NA
M-*dptS69*	0.0801	0.2515	0.095	0.9423	0.0119	0.9995	0.3653
Day 20 L. brevis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9999	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.9998	0.9808	NA	NA	NA	NA	NA
F-*dptS69*	1	0.9997	0.9999	NA	NA	NA	NA
M-*imd-*	0.0045	2.00E-04	0.0175	0.0056	NA	NA	NA
M-*Δdpt*	0.9469	0.9854	0.7085	0.9241	0	NA	NA
M-*dptS69R*	0.9981	1	0.9528	0.9962	6.00E-04	0.9999	NA
M-*dptS69*	0.954	0.9876	0.7694	0.9363	1.00E-04	1	0.9998
Day 2 A. tropicalis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9999	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.9981	0.9373	NA	NA	NA	NA	NA
F-*dptS69*	0.9993	0.9626	1	NA	NA	NA	NA
M-*imd-*	2.06E-01	0.0304	0.5635	0.5008	NA	NA	NA
M-*Δdpt*	0.5631	0.1075	0.9417	0.9084	0.9672	NA	NA
M-*dptS69R*	0.1343	0.0157	0.4295	3.72E-01	1	0.9048	NA
M-*dptS69*	0.9994	0.9634	1	1.00E+00	0.4983	0.9069	0.3698
Day 10 A. tropicalis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.5974	NA	NA	NA	NA	NA	NA
F-*dptS69R*	1	0.2639	NA	NA	NA	NA	NA
F-*dptS69*	0.812	1	0.5422	NA	NA	NA	NA
M-*imd-*	0.9993	0.1249	1	0.3359	NA	NA	NA
M-*Δdpt*	0.004	0.0796	2.00E-04	0.1109	1.00E-04	NA	NA
M-*dptS69R*	0.9998	0.7771	9.94E-01	0.9394	0.9524	0.0035	NA
M-*dptS69*	<2e-16	2.00E-04	<2e-16	5.00E-04	<2e-16	0.2145	<2e-16
Day 20 A. tropicalis							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.9956	NA	NA	NA	NA	NA	NA
F-*dptS69R*	1	0.9988	NA	NA	NA	NA	NA
F-*dptS69*	1	0.998	1	NA	NA	NA	NA
M-*imd-*	0.9999	0.6771	0.9677	0.9743	NA	NA	NA
M-*Δdpt*	0.1447	3.77E-02	0.0201	1.77E-02	8.00E-04	NA	NA
M-*dptS69R*	1	9.99E-01	1	1	0.9677	0.0201	NA
M-*dptS69*	4.90E-03	1.00E-04	1.00E-04	1.00E-04	<2e-16	0.3458	1.00E-04

1025 **Table S6. P-values from Tukey HSD for bacterial load from poly-association with *L. plantarum* and *P.***
1026 ***rettgeri*.** P-values less than 0.05 are underlined.
1027

Day 2 *L. plantarum*							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.2226	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.0101	0.6004	NA	NA	NA	NA	NA
F-*dptS69*	<2e-16	2.00E-04	0.1471	NA	NA	NA	NA
M-*imd-*	4.21E-01	1	0.7283	0.0017	NA	NA	NA
M-*Δdpt*	0.0031	4.83E-01	1	0.0344	0.6898	NA	NA
M-*dptS69R*	<2e-16	6.00E-04	0.2442	1	0.0038	0.0725	NA
M-*dptS69*	<2e-16	4.00E-04	0.2095	1	0.0029	0.0577	1
Day 10 *L. plantarum*							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.2226	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.0101	0.6004	NA	NA	NA	NA	NA
F-*dptS69*	<2e-16	2.00E-04	0.1471	NA	NA	NA	NA
M-*imd-*	4.21E-01	1	0.7283	0.0017	NA	NA	NA
M-*Δdpt*	0.0031	4.83E-01	1	0.0344	0.6898	NA	NA
M-*dptS69R*	<2e-16	6.00E-04	0.2442	1	0.0038	0.0725	NA
M-*dptS69*	<2e-16	4.00E-04	0.2095	1	0.0029	0.0577	1
Day 20 *L. plantarum* (no imd females)							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	NA	NA	NA	NA	NA	NA	NA
F-*dptS69R*	NA	0.831	NA	NA	NA	NA	NA
F-*dptS69*	NA	0.9883	0.9977	NA	NA	NA	NA
M-*imd-*	NA	0.5334	0.9998	0.9429	NA	NA	NA
M-*Δdpt*	NA	0.9999	0.484	0.857	0.1772	NA	NA
M-*dptS69R*	NA	0.9202	1	0.9999	0.9943	0.6188	NA
M-*dptS69*	NA	0.9999	0.5746	0.8987	0.2615	1	0.7064
Day 2 *P. rettgeri*							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	0.0784	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.1199	1	NA	NA	NA	NA	NA
F-*dptS69*	0.434	0.9992	0.9966	NA	NA	NA	NA
M-*imd-*	0.8069	0.912	0.8957	0.9988	NA	NA	NA
M-*Δdpt*	0.0063	0.9457	0.9972	0.8097	0.3815	NA	NA
M-*dptS69R*	0.131	1	1	0.9977	0.9101	0.9957	NA
M-*dptS69*	0.0348	0.9902	0.9996	0.9254	0.6186	1	0.9993
Day 10 *P. rettgeri*							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	1	NA	NA	NA	NA	NA	NA
F-*dptS69R*	0.993	0.9967	NA	NA	NA	NA	NA
F-*dptS69*	0.9835	0.9901	1	NA	NA	NA	NA
M-*imd-*	0.998	0.9994	1	1	NA	NA	NA
M-*Δdpt*	1	0.9997	0.9485	0.9085	0.9773	NA	NA
M-*dptS69R*	1	1	0.9998	0.9992	1	0.9981	NA
M-*dptS69*	0.9911	0.9954	1	1	1	0.9393	0.9998
Day 20 *P. rettgeri* (no imd females)							
	F-*imd-*	F-*Δdpt*	F-*dptS69R*	F-*dptS69*	M-*imd-*	M-*Δdpt*	M-*dptS69R*
F-*Δdpt*	NA	NA	NA	NA	NA	NA	NA
F-*dptS69R*	NA	0.9927	NA	NA	NA	NA	NA
F-*dptS69*	NA	0.9971	0.8027	NA	NA	NA	NA
M-*imd-*	NA	0.9999	0.911	1	NA	NA	NA
M-*Δdpt*	NA	9.64E-01	1	0.5715	0.7503	NA	NA
M-*dptS69R*	NA	1	0.9896	0.9966	0.9998	0.9478	NA
M-*dptS69*	NA	0.9991	1	0.8929	0.9652	0.9998	0.9986

1028

1029 **Table S7. Desiccation resistance Tukey HSD p-values.** P-values less than 0.05 are underlined. "A" refers to flies
1030 reared axenically, "C" refers to flies reared on a conventional diet.
1031

	A *dptS69*	A *Δdpt*	A *dptS69R*	A *imd-*	A OreR	A w1118	C *dptS69*	C *Δdpt*	C *dptS69R*	C *imd-*	C OreR
A *Δdpt*	0.9999	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
A *dptS69R*	0.3541	0.9978	NA	NA	NA	NA	NA	NA	NA	NA	NA
A *imd-*	0.9195	0.8332	0.003	NA	NA	NA	NA	NA	NA	NA	NA
A OreR	1	1	0.5898	0.8561	NA	NA	NA	NA	NA	NA	NA
A w1118	<2e-16	7.00E-04	<2e-16	0.007	<2e-16	NA	NA	NA	NA	NA	NA
C *dptS69*	<2e-16	0.1208	0.1473	<2e-16	1.00E-04	<2e-16	NA	NA	NA	NA	NA
C *Δdpt*	<2e-16	5.00E-04	2.00E-04	<2e-16	<2e-16	<2e-16	0.3672	NA	NA	NA	NA
C *dptS69R*	<2e-16	0.0193	0.0086	<2e-16	<2e-16	<2e-16	0.9995	0.8062	NA	NA	NA
C *imd-*	0.0057	0.7281	0.963	<2e-16	0.0242	<2e-16	0.934	0.013	0.3953	NA	NA
C OreR	0.0024	0.5452	0.8351	<2e-16	0.0105	<2e-16	0.9979	0.0653	0.8078	1	NA
C w1118	0.9996	1	0.8729	0.3974	1	<2e-16	3.00E-04	<2e-16	<2e-16	0.0781	0.0352

1032

1033 **Table S8. Starvation resistance Tukey HSD p-values.** P-values less than 0.05 are underlined. "A" refers to flies
1034 reared axenically, "C" refers to flies reared on a conventional diet.
1035

	A *dptS69*	A *Δdpt*	A *dptS69R*	A *imd-*	A OreR	A w1118	C *dptS69*	C *Δdpt*	C *dptS69R*	C *imd-*	C OreR
A *Δdpt*	0.0295	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
A *dptS69R*	<2e-16	0.0022	NA	NA	NA	NA	NA	NA	NA	NA	NA
A *imd-*	<2e-16	<2e-16	0.9288	NA	NA	NA	NA	NA	NA	NA	NA
A OreR	0.0043	1	8.00E-04	<2e-16	NA	NA	NA	NA	NA	NA	NA
A W1118	<2e-16	<2e-16	<2e-16	0.0038	<2e-16	NA	NA	NA	NA	NA	NA
C *dptS69*	5.00E-04	<2e-16	<2e-16	<2e-16	<2e-16	<2e-16	NA	NA	NA	NA	NA
C *Δdpt*	0.0055	<2e-16	<2e-16	<2e-16	<2e-16	<2e-16	1	NA	NA	NA	NA
C *dptS69R*	<2e-16	0.0614	0.998	0.3139	0.0407	<2e-16	<2e-16	<2e-16	NA	NA	NA
C *imd-*	2.00E-04	0.9988	0.0306	<2e-16	0.9995	<2e-16	<2e-16	<2e-16	0.3851	NA	NA
C OreR	0.7443	0.9473	<2e-16	<2e-16	0.828	<2e-16	<2e-16	<2e-16	1.00E-04	0.325	NA
C w1118	<2e-16	<2e-16	1.00E-04	0.0374	0	1	<2e-16	<2e-16	<2e-16	<2e-16	<2e-16

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1039
1040 **Table S9. Conventional longevity Cox proportional hazards regression p-values.** P-values less than 0.05 are
1041 underlined. "F" and "M" refer to males and females, respectively; "het" refers to heterozygotes for serine/arginine.
1042

	F het	F *imd-*	F *Δdpt*	F *dptS69R*	F *dptS69*	M het	M *imd-*	M *Δdpt*	M *dptS69R*
F *imd-*	1	NA	NA	NA	NA	NA	NA	NA	NA
F *Δdpt*	1	1	NA	NA	NA	NA	NA	NA	NA
F *dptS69R*	<2e-16	<2e-16	<2e-16	NA	NA	NA	NA	NA	NA
F *dptS69*	<2e-16	3.00E-04	<2e-16	0.6785	NA	NA	NA	NA	NA
M het	<2e-16	<2e-16	<2e-16	0.9797	0.0443	NA	NA	NA	NA
M *imd-*	0.1346	0.3182	0.0498	0.0025	0.5097	0	NA	NA	NA
M *Δdpt*	<2e-16	<2e-16	<2e-16	0.948	0.9977	0.1163	0.0321	NA	NA
M *dptS69R*	<2e-16	<2e-16	<2e-16	0.0622	<2e-16	0.4311	<2e-16	<2e-16	NA
M *dptS69*	<2e-16	<2e-16	<2e-16	0.9984	0.9882	0.5841	0.0492	1	0.0043

1043

Table S10. Genotyped wild caught *D. melanogaster.*

Wolbachia/Sex	SS	SR	RR
Wolbachia+ female	203	64	8
Wolbachia+ male	369	77	9
Wolbachia- female	68	21	2
Wolbachia- male	102	31	1
Total	742	193	20

Supplemental Figures

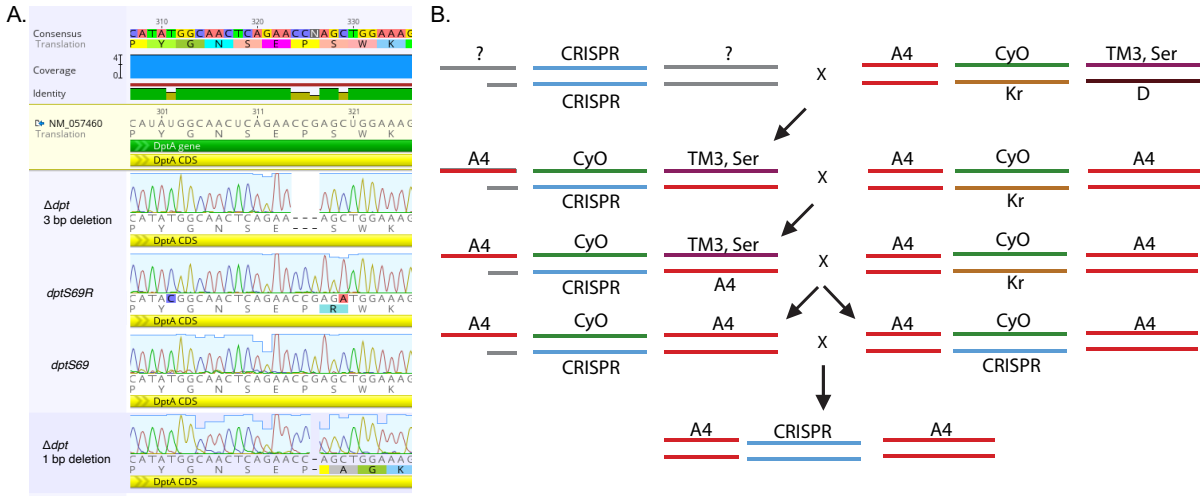

Figure S1. CRISPR/Cas9 genome editing of the *dpt* locus

A) Alignment of CRISPR/Cas9 genome edited lines. CRISPR/Cas9 genome edited lines aligned to consensus sequence. Genotype/line name is indicated on the left-hand side of the figure. B) Crossing schematic for CRISPR clean up. A modified Bloomington stock # 7198 was used for CRISPR injections. The line contained *CyO*/*Kruppel* balanced on the 2nd chromosome, and *serrate*/*Dichaete* balanced on the 3rd chromosome. Our version, 7198^{A4}, was provided by Stuart Macdonald and has the DSPR (King et al. 2012) A4 line's X chromosome instead of the *w*[*] from the original 7198.

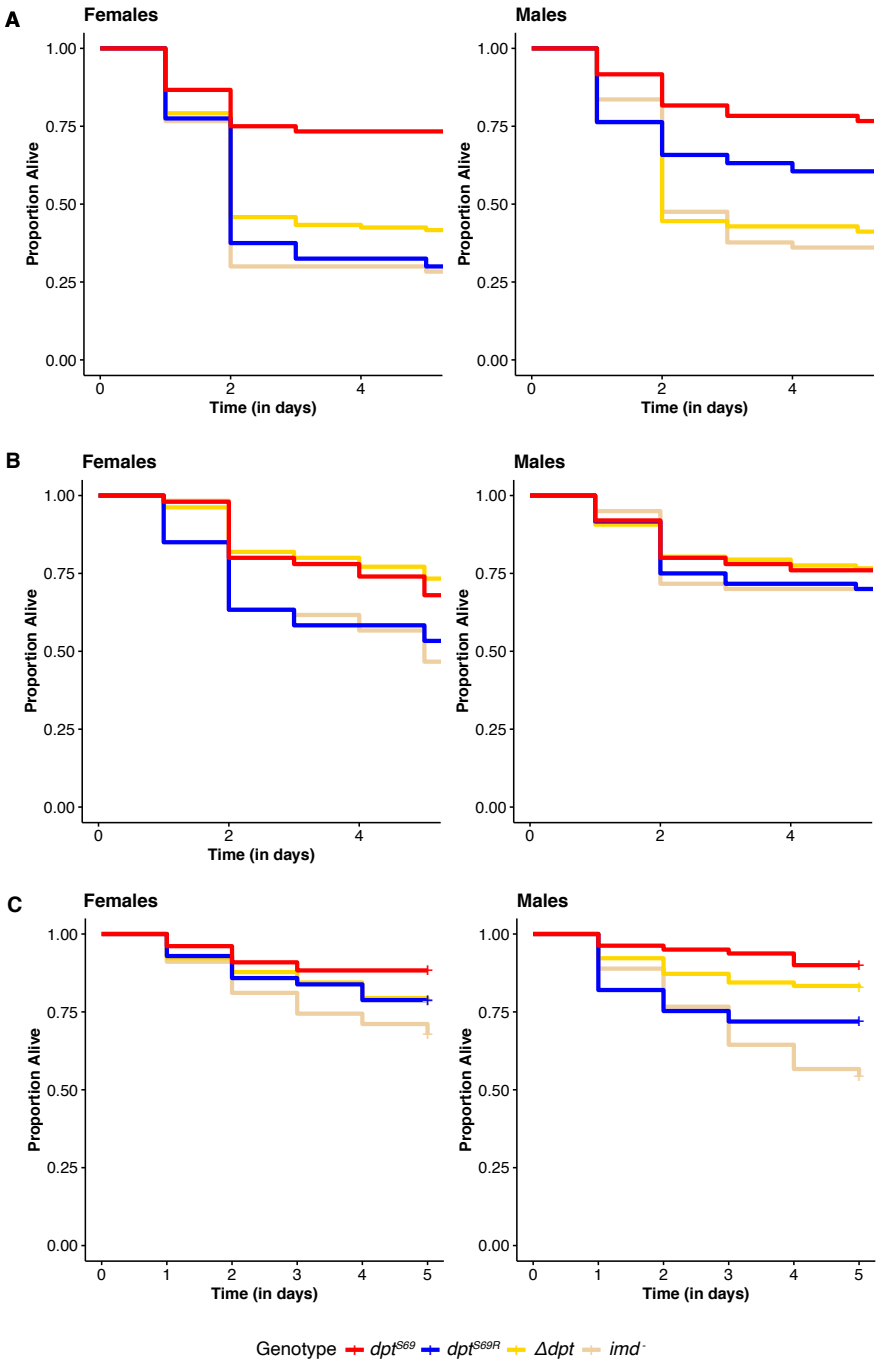

Figure S2. Male vs. female survival after systemic infection

Systemic infections were repeated with females and males. A) *P. rettgeri* and B) *E. faecalis* are normal systemic infection bacteria in *Drosophila*. C) *L. plantarum* was used to test differences in systemic based on sex based on the results from gnotobiotic longevity.

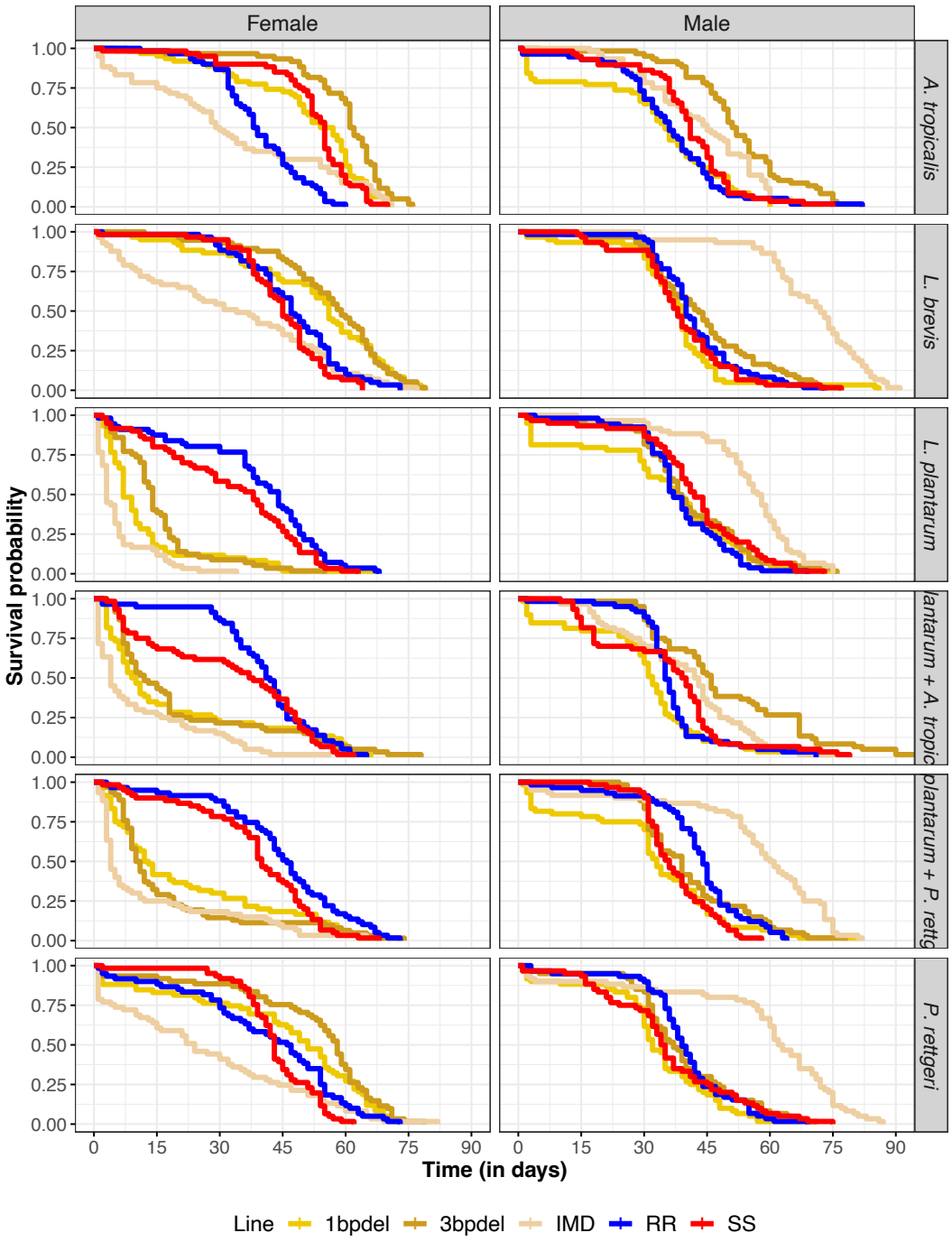

Figure S3. Gnotobiotic longevity
Survival plots of all bacteria. Dpt null lines have been separated into the two lines used, 1 base pair deletion and 3 bp deletion.

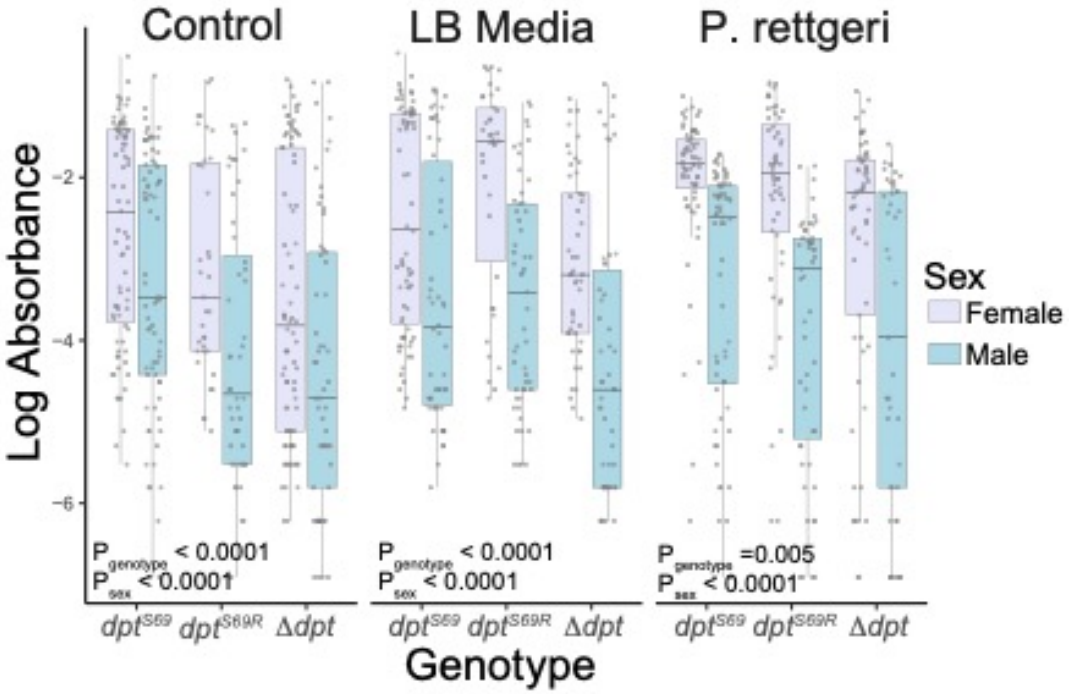

Figure S4. Feeding Rates

Female flies eat more food (as measured by blue dye ingestion) than males, but genotype also influences feeding. Each genotype has more than 100 individuals per treatment per sex. Control refers to no added media, LB Media is the addition of sterile MB, and *P. rettgeri* is the addition of *P. rettgeri* at a concentration of OD_{600} = 15 (±1). Absorbance was measured at 630nm.

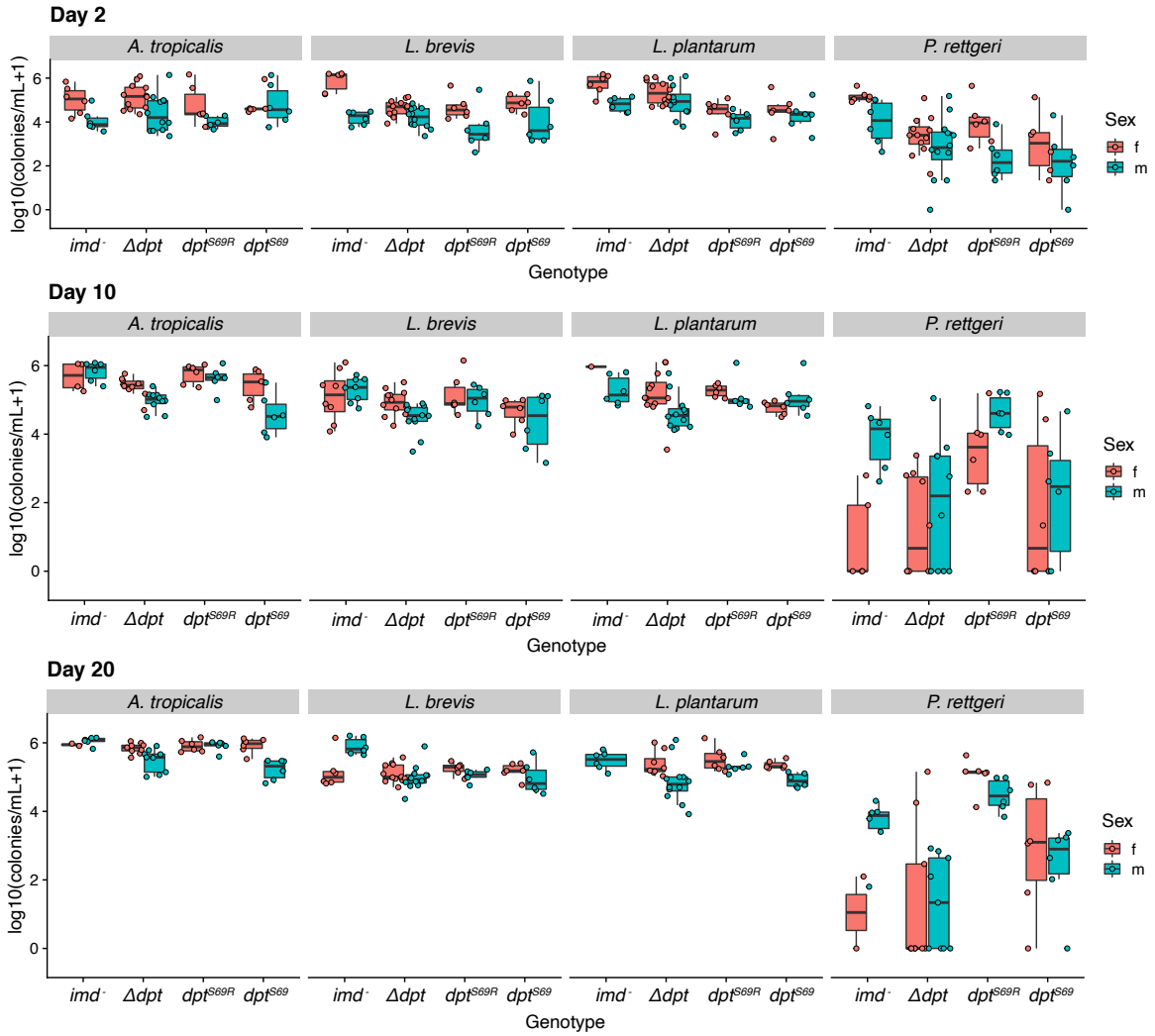

Figure S5. Bacterial load at day 2-, 10-, and 20-days post feeding
All bacteria were plated on MRS media except for *P. rettgeri* that was plated on LB media to grow colonies. Significance can be found in Table 2.S6. Individual points represent a pool of 3 flies. There is no *imd*-female bar for *L. plantarum* on day 20 because all female flies had died at this timepoint.

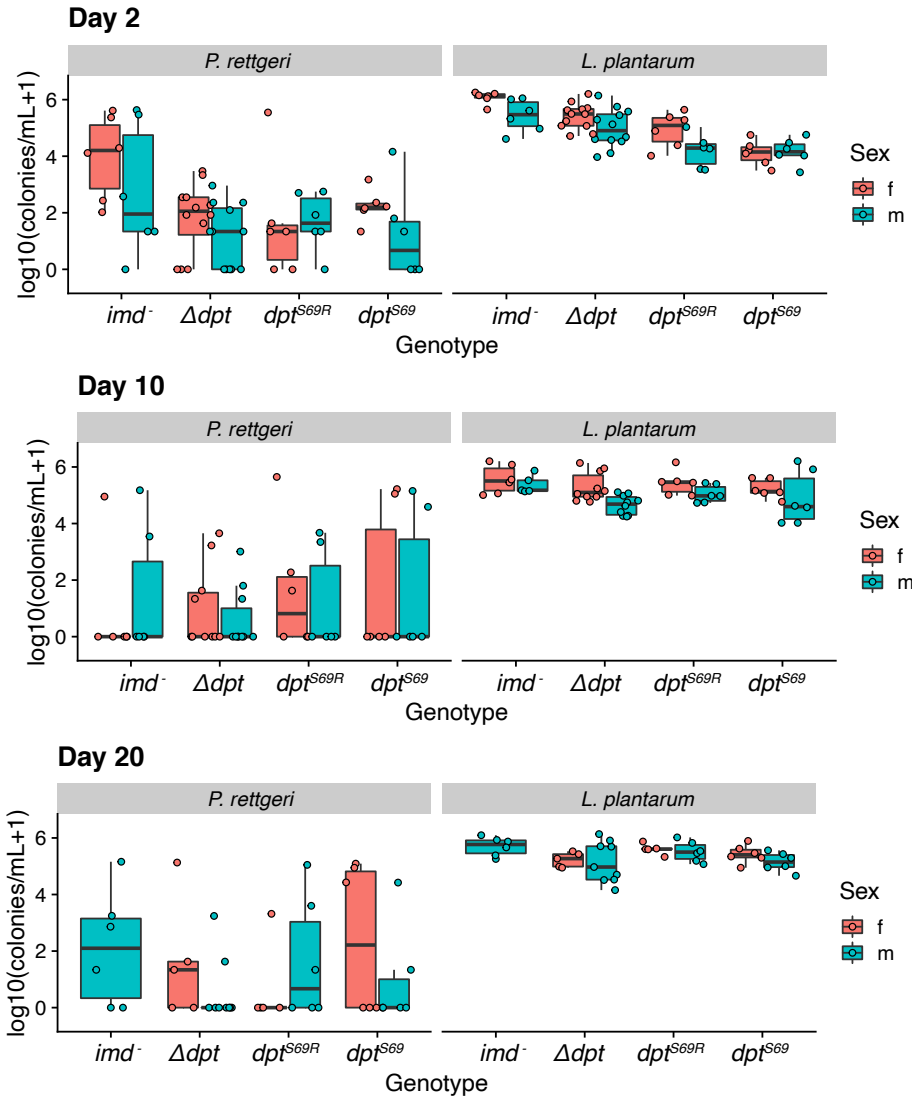

Figure S6. Bacterial load of *L. plantarum* and *P. rettgeri* poly-association
The flies were co-feed with *L. plantarum* and *P. rettgeri* and plated on MRS and LB respectively to select for the different bacteria. There is no *imd*- female bar on day 20 because all flies had died at this timepoint.

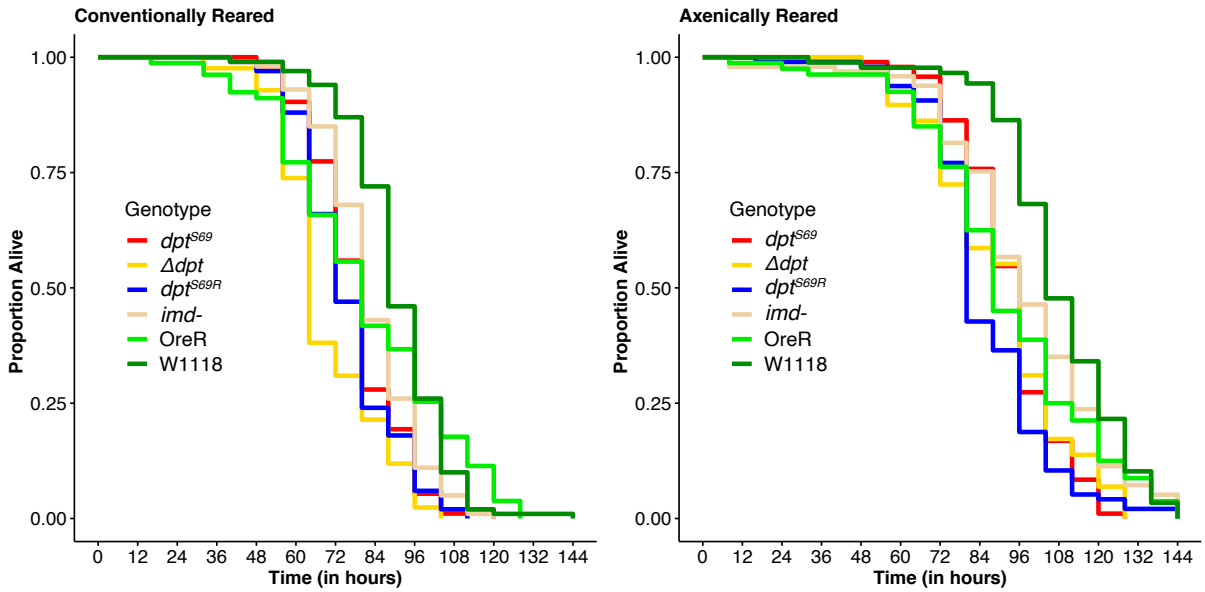

Figure S7. Desiccation stress survival with additional wildtype lines (OreR and W1118)
OreR and W1118 are standard *Drosophila melanogaster* lab stocks. They are both homozygous serine but have different resistance to desiccation stress than dpt^{S69}.

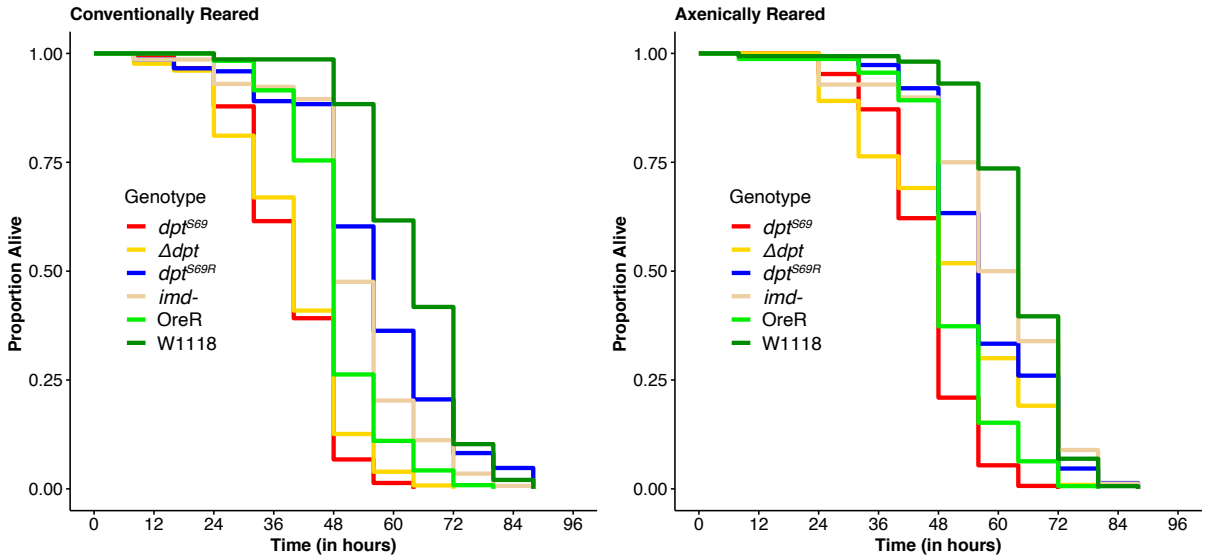

Figure S8. Starvation with additional wildtype lines (OreR and w1118)
OreR and W1118 are standard *Drosophila melanogaster* lab stocks. They are both homozygous serine but have different resistance to starvation stress than dpt^{S69}.

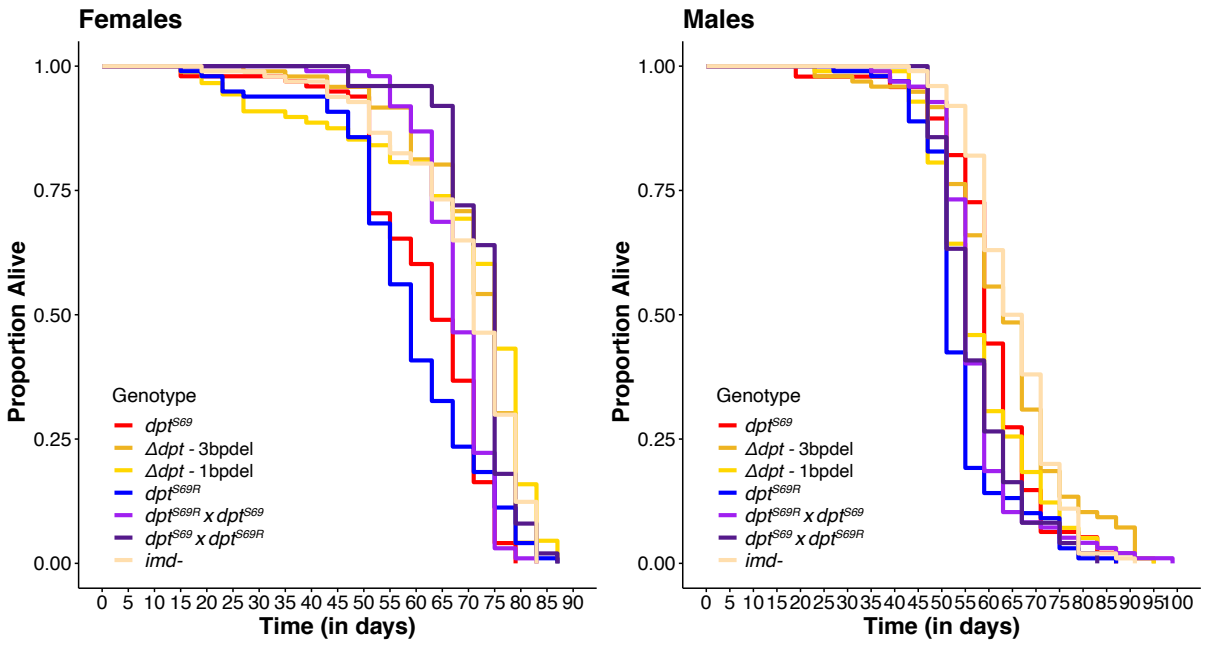

Figure S9. Longevity with separated *Δdpt* lines and heterozygotes

Two *Δdpt* lines were used. One with a 1 base pair deletion and the other with a 3 base pair deletion. These lines were combined for ease of viewing in Fig 4C. Heterozygotes were made with reciprocal crosses